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Glutamate signaling through the Kainate Receptor Enhances Human Immunoglobulin Production

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Abstract

CD23 is implicated as a regulator of IgE synthesis. A soluble form of CD23 (sCD23) is released following cleavage by ADAM10 and enhanced sCD23 is correlated with increased IgE. In the CNS, signaling through the kainate receptor (KAR) increases ADAM10. In B cells, activation of KARs produced a significant increase in ADAM10 and sCD23 release as well as an increase in B cell proliferation and immunoglobulin production. In addition, ADAM10 inhibitors reduce IgE synthesis from in vitro cultures of human B cells. Thus, we report for the first time the unique presence of the kainate receptor in B cells and that activation of KARs could serve as a novel mechanism for enhancing B cell activation.

Keywords

Glutamate; B cell; Kainate receptor; CD23; Immunoglobulin; ADAM10

Introduction

CD23 (FCεRII) is a type II integral membrane protein and the low affinity receptor for IgE. Through studies using knock-out and transgenic animals, CD23 has been implicated as a natural, negative regulator of IgE production (Texido et al., 1994; Yu et al., 1994). Although the mechanism remains unclear, interactions with cell surface CD21 as well as direct signaling by the membrane form of CD23 have both been hypothesized to be responsible for IgE modulation. A soluble monomeric form of CD23 (sCD23) is released following proteolytic cleavage by a disintegrin and metalloprotease 10 (ADAM10) (Weskamp et al., 2006). Enhanced CD23 cleavage has been shown to correlate with increased IgE production in both mouse and human (Ford et al., 2006; Saxon et al., 1990). In addition to its effects on allergic disease, sCD23 has been linked to the activation of macrophages, via interaction with CD11b/CD18 or CD11c/CD18, resulting in the release of pro-inflammatory mediators and the onset of inflammatory disease (Lecoanet-Henchoz et al., 1995).

In view of the recent demonstration that ADAM10 is the primary CD23 sheddase, we searched for agents that would modify ADAM10 activity. The overall purpose was to test

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the hypothesis that ADAM10 modulation would, by virtue being the CD23 sheddase, result in IgE modulation. Ortiz *et al.* showed that when a specific type of glutamate receptor, namely the kainate receptor (KAR), was stimulated with its ligand, ADAM10 mRNA increased (Ortiz et al., 2005). KARs are one of three types of multi-subunit, ionotropic glutamate receptors which are named based upon their preferred pharmacological ligand: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), and kainic acid (KA). KARs are the most recently identified of the three and have been shown to be widely expressed in the central nervous system (CNS) (Chittajallu et al., 1999; Lerma, 2006), however, little is reported on their presence outside the CNS. Kainic acid, a chemical first isolated from the red algae *Digenea simplex*, is a potent agonist of KARs and is a widely used for the generation of epilepsy in laboratory rodent models due to its ability to cause neuro-inflammation following epilepsy induction (Oprica et al., 2006; Engel et al., 2009; Ramsdell and Stafstrom, 2009; Gupta et al., 2009; Zemlyak et al., 2009).

Glutamate, the major excitatory neurotransmitter in the CNS has recently been implicated in a variety of diseases. For example, it has been shown that patients with certain cancers (Eck et al., 1990), human immunodeficiency virus (HIV) (Eck et al., 1989), epilepsy (Rainesalo et al., 2004), autism (Aitken, 2008), and certain autoimmune illnesses such as rheumatoid arthritis (RA) (McNearney et al., 2000), and systemic lupus erythematosus (SLE) (West, 2007) all have elevated levels of glutamate in the periphery. Interestingly, autoimmune disease treatments which include corticosteroid use can also increase peripheral glutamate levels (Borsody and Coco, 2001; Raber, 1998; Eck et al., 1990). While glutamate receptor signaling has been examined in T cells (Ganor et al., 2003a) and macrophages (Boldyrev et al., 2004), there are currently no published observations on the effects of glutamatergic stimuli on B cells. We report that human B cells do indeed express the kainate receptor. In keeping with the Ortiz study (Ortiz et al., 2005), KAR activation was found to increased ADAM10 expression and activity, as measured by sCD23 release. A significant increase in B cell proliferation and Ig production was also seen with both purified B cells and PBMC. The implications of this finding for human allergic and autoimmune diseases are discussed.

Materials and Methods

Media, Reagents, and Cell Lines

All cells were grown in complete culture medium as indicated (CRPMI-10 or CDMEM-10; RPMI-1640 or Dulbecco's Modified Eagle Medium containing 10% heat inactivated (56°C, 30 min) fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μg/ml amphotericin B, 50μM 2-mercaptoethanol and 20mM HEPES buffer (all from Invitrogen Carlsbad, CA)). All lines are kept in confluent culture under log phase growth in complete culture medium at 37 °C in humidified air with 5% CO_2. Kainic Acid (KA), dimethylsulfoxide (DMSO), L-glutamic acid (Glu), and antagonists (topiramate (TPM), NS102 and NBQX) were all purchased from Sigma (St. Louis, MO). Human IL-21 and mouse antihuman CD40 (clone G28-5) (American Type Culture Collection, (ATCC), Manassas, VA) were generated in our laboratory as previously described (Caven et al., 2005a). rhIL-4 was purchased from R&D Systems (Minneapolis, MN). The ADAM10 selective inhibitor INCB008765 was kindly provided by Incyte Corporation, Wilmington, DE (Fridman et al., 2007; Zhou et al., 2006).

Human Cells

Human tonsils were obtained from routine tonsillectomies at Henrico Doctors Hospital (Richmond, VA) or the VCU Tissue Data Acquisition and Analysis Core (TDAAC). Tonsils were placed in media supplemented with antibiotics and mechanically disrupted using a

Seward Stomacher 80 Biomaster Lab Blender (Brinkmann, Westbury, NY) at normal speed for 60 seconds. To obtain a single cell suspension, the resulting product was underlayed with Ficoll–Hypaque (GE Healthcare Piscataway, NJ). Following centrifugation (20 min at 400 × g), the cells at the interface were removed and washed in PBS. To isolate B cells, the tonsilar cells were incubated with a FITC-anti-human IgD (BD Pharmingen San Diego, CA) for 30 min on ice and B cells were isolated by using the Miltenyi anti-FITC Microbeads, per manufacturer's instructions (Miltenyi Biotec Auburn, CA) Final B cell preparations were >95% pure IgD+ by FACS analysis.

Alternatively when whole PBMC was used, buffy coats were obtained from the Virginia Blood Service Center (Richmond, VA). PBMC were isolated by Ficoll gradient density centrifugation. All human studies research was performed in accordance to the Virginia Commonwealth University Institution Review Board per approved protocols.

RT-PCR and qPCR

RNA was isolated via standard Trizol (Invitrogen) purification protocol and the Access Quick RT-PCR kit (Promega, Madison WI) was used with gene specific primers to examine for the presence of kainate receptor subunits and their transcript variants. Primers were designed using VectorNTI software (Invitrogen). Ready-made primers® for G3PDH and all custom made primers for all sequences were synthesized by Integrated DNA Technologies (Coralville, IA). qPCR experiments were performed by the nucleic acid core in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, Ca) using the TaqMan® One Step PCR Master Mix Reagents Kit. All the samples were tested in triplicate using the following conditions: 48°C/30min; 95°C/10min; and 40 cycles of 95°C/15sec and 60°C/1min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The probes and primers for ADAM10 (#Hs00153853_m1) were purchased from ABI. Human beta actin primers were from the Pre-developed TaqMan®Assay Reagents and were used as endogenous control. Relative ADAM10 mRNA is determined as a relative ratio based upon beta-actin.

Western Blot

Five million cells were lysed in Hepes Buffered Saline (HBS) with 1% NP-40 on ice for 10 minutes. Nuclei were removed by centrifugation and cytosolic proteins were treated with SDS buffer and heated at 70°C for 10 minutes. Proteins were run on an MES NuPage gel (Invitrogen) and then transferred to nitrocellulose. Blots were stained with a rabbit polyclonal antibody against the human kainate receptor subunit GluK4 (known as *GRIK4* in Genbank) (Chemicon AB5649). Detection was performed with a goat anti-rabbit IgG HRP and chemiluminescence was performed with SuperSignal West Pico Chemiluminescence Substrate (Pierce). To ensure equal loading, Ponceau S (Sigma) staining was performed.

Cell Surface Phenotyping

All cells were tested for cell surface antigen expression by direct immunofluorescence and flow cytometric analysis. Briefly, 1×10^6 cells were stained in 100μl volumes with rabbit anti-human GluK4 (Chemicon) for primary incubation for 30 min at 4°C. After washing, cells were then stained with a PE labeled goat anti-rabbit IgG (BD Pharmingen San Diego, CA). After 30 min/4°C and washing, the cells were examined using a Cytomics FC500 Flow Cytometer and data was analyzed using CXP software (Beckman Coulter Fullerton, CA). PI was used to exclude dead cells from the analysis.

Soluble CD23 Release Assay

For these studies, the $CD23^+$ human B cell line RPMI8866 was grown in the presence or absence of 5mM KA or Glu for 24 hours in CDMEM10 at a concentration of 1×10^6 cells/mL. For antagonist studies, prior to the addition of KA or Glu cells were pre-treated with 50μM vehicle (DMSO), NBQX, NS102, or TPM for one hour. After incubation, cell free supernatants were harvested and sCD23 levels determined by ELISA. When primary cells were assayed, cells were cultured with 10 ng/mL IL-4, 1 μg/mL anti-CD40, and 200ng/mL Il-21 in complete culture media in the presence or absence of 5mM glutamate. Fortyeight hours later cell free supernatants were harvested for ELISA.

Culture Conditions for Immunoglobulin Analysis

Primary B cells or PBMC were cultured in the presence of 10 ng/mL IL-4 and 1 μg/mL anti-CD40 in complete culture media in the presence or absence of 5mM KA or 5mM Glu. When indicated, 200ng/mL IL-21 was also added. For antagonism studies, prior to culture primary cells were treated with 10μM vehicle (DMSO), NBQX, NS102, or TPM. After 14 days of culture, cell free supernatants were analyzed via ELISA for Ig levels. As cells are grown at various densities, line graphs represent Ig production as compared to cell density. When expressed as a bar graph, data represents the cell concentration in which maximum Ig production was observed for the particular isotype displayed.

To determine the effect of ADAM10 inhibition on Ig production, purified human B cells were cultured with 10 ng/mL IL-4, 1 μg/mL anti-CD40, and 200ng/mL IL-21 in complete culture media in the presence of DMSO as vehicle control or 10μM ADAM10 specific inhibitor. Five days later, cell free supernatants were harvested for soluble CD23 release and fourteen days later, cell free supernatants were analyzed for Ig production.

ELISAs

Human sCD23 ELISA was measured using a standard sandwich ELISA approach, using a mouse anti-CD23 (Clone BU38) coating antibody and sheep anti-CD23 (both from The Binding Site Birmingham, UK). Detection is performed with a goat anti-sheep IgG tagged with HRP (Southern Biotech Birmingham AL). Determination of human IgE levels utilized a monoclonal mouse anti-human IgE antibody (clone 4.15) as a capture. Samples and standards were detected using a rabbit anti-human IgE-HRP (Southern Biotech) diluted in PBS/10% FBS. Human IgG or IgM were detected using a goat anti-human IgG or IgM followed by detection with a goat anti-human IgG or IgM tagged with HRP (All from Southern Biotech). Standards for the IgG and IgM ELISAs were purchased from Sigma. IgE standards were purified from JW8 hybridoma cells as previously described (Caven et al., 2005a). All assays utilized TMB substrate (BD Pharmingen San Diego, CA) and the reactions stopped with 0.18M H_2SO_4. Plates are read at a wavelength of 450nm on a SpectraMax 250 and data analyzed using SOFTmax PRO 3.1.2 software (Molecular Devices, Los Angeles, CA).

Proliferation Assay

Primary B cells were cultured with 10 ng/mL IL-4, 1 μg/mL anti-CD40, and 200ng/mL IL-21 in CDMEM-10 in the presence or absence of 5mM glutamate at a concentration of 150,000 cells/well in sterile 96-well culture plates. When PBMC is used the cell concentration is 100,000 cells/well. Prior to the addition of glutamate, cells were pretreated for 1 hour with 10μM vehicle (DMSO) or NS102. After 96 hours of growth, a 24 hr pulse [H^3]-thymidine (Perkin Elmer) was used. Plates were then harvested using a Filtermate cell harvester onto GFC plates. Assays were read using a Topcount Plate Counter (Perkin Elmer, Waltham, MA).

Analysis of cell division

Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Eugene, OR) was prepared according to the manufacturer's recommendations. Resting B cells were washed and re-suspended at 5×10^6cells/mL in PBS. CFSE was then added at the manufacturer's recommended dilution and incubated in the dark at room temp for 5 minutes. Reaction was quenched by the addition of ice cold FBS and cells were then washed and plated at 1×10^4 cells/200 μl in a 96 well plate containing 10 ng/mL IL-4, 1 μg/mL anti-CD40, and 200ng/mL IL-21 in the presence or absence of glutamate. After 5 days of culture, cells were harvested and analyzed by flow cytometry using a BD Canto. Analysis was performed using FCS Express software V3.

Data analysis

Data are summarized as mean ± Standard error (SE). The statistical analysis of the results was performed by the student's *t* test. A p-value of <0.05 was considered significant. When primary cells are used, data is one representative donor but all assays have been performed using a minimum of three donors with similar results.

Results

Kainate receptors are present on human B cells

KARs are multi-subunit receptors consisting of four distinct subunits. The gene names for these subunit are designated as *GRIK1-5*, whereas the protein names are GluK1, GluK2, GluK3, GluK4, and GluK5 (Collingridge et al., 2009). Thus, to form a functional receptor, KARs must consist of GluK1 or GluK2 in combination with GluK3, GluK4, and GluK5. Two of the genes that encode subunit GluK1 (*GRIK1*) and GluK2 (*GRIK2*), can undergo specific RNA editing and thus result in two transcript variants (TVs). From the data shown, we observe the presence of four subunit gene products, *GRIK2, GRIK3, GRIK4*, and *GRIK5*, thus, it is evident that message for KARs do exist in human B cells (Figure 1A). It appears that the immune type of kainate receptor is the GluK2 (*GRIK2*) containing receptor as evidenced by the presence of this transcript. Furthermore, the fact that four subunits are present would indicate the potential presence of a functionally active receptor. We confirmed our RT-PCR data by showing protein expression of the kainate receptor subunit GluK4 (*GRIK4*) by Western Blot analysis, using B cell lines and primary human lymphocytes (Figure 1B). We focused on the presence of GluK4 (*GRIK4*) as it is required for ligand binding. In addition, flow cytometric analysis confirmed that KARs are cell surface expressed on both RPMI8866 (data not shown) and primary human leukocytes from PBMC and tonsils (Figure 1C).

KAR activation increases ADAM10 mRNA and activity

Based upon the publication by Ortiz *et al.*., KAR stimulation in the CNS led to an increase in ADAM10 mRNA. In order to determine if kainic acid (KA), an exogenous ligand, or glutamate (Glu), an endogenous ligand, has the ability to increase ADAM10 message levels in the human immune system, purified human B cells were cultured in the presence of KA or Glu for 30 minutes and then RNA analyzed by qPCR for ADAM10 expression. As evidenced in Figure 2A, there is a significant increase in the mRNA levels for ADAM10 after KAR activation in primary B cells. In keeping with ADAM10's newly identified role as the CD23 sheddase, it was anticipated that KAR activation would increase soluble CD23 release. For these studies, the $CD23^+$ human B cell line RPMI8866 was utilized and experiment was performed as outlined in Materials and Methods. As shown in Figure 2B, a significant elevation in the amount of sCD23 released is seen following KAR activation by either KA or glutamate. We also wanted to determine if this same increase in sCD23 release

is observed in primary human B cells. B cells were cultured in the presence of IL-4, anti-CD40, IL-21, +/− 5mM glutamate for 48 hours. The reason that primary cells are cultured longer than RPMI8866 is that CD23 first needs to be up-regulated, which takes approximately 24 hours, before it can be cleaved. Figure 2C shows that sCD23 release is also appreciably increased in primary human B cells in the presence of glutamate.

As stated earlier, three types of ionotropic glutamate receptors, NMDAR, AMPAR, and KAR exist. High doses of kainic acid can activate AMPA receptors and both AMPA and NMDA receptors have been described on other immune cells (Lerma et al., 2001; Ganor et al., 2003b), (Boldyrev et al., 2005). Therefore, receptor antagonists were used to confirm that the KAR was indeed responsible for the enhanced CD23 cleavage. NS102 is a specific KAR antagonist (Du et al., 2009; Verdoorn et al., 1994), NBQX is antagonist of the AMPA receptor (Hou et al., 2009) and topiramate (TPM) is a NMDA antagonist (Rawls et al., 2009). The soluble CD23 release assays were performed as before, except the RPMI8866 cells were incubated with 50μM of the antagonist for 1hr prior to the addition of KA or Glu. Figure 2D shows that none of the antagonists have any effect on baseline sCD23 release. Furthermore, only NS102, the KAR specific antagonist, could prevent the significant increase observed in the presence of KAR stimulation. Verdoorn *et al.*. showed that NS102 selectively antagonizes GluK2 containing KARs (Verdoorn et al., 1994). This fact is in agreement with our data in that we observe the GluK2 subtype of the KAR receptor in the immune system and indeed NS102 does block. This shows that this phenomenon is KAR specific due to the fact that no change is observed in the presence of the AMPAR or NMDAR antagonists. Thus, we are confident that the observations made are a direct result of KAR activation on the human B cells.

KAR activation increases IgE synthesis

Because there was a significant increase in sCD23 released from the cell surface, the natural extension of these studies was to look at IgE synthesis as CD23 is a regulator of IgE production. Primary human B cells were stimulated with IL-4 and anti-CD40 to stimulate IgE production and cultured in the presence or absence of KA. When KA is present in the media, there is a strong and statistically significant increase in the amount of IgE produced (Figure 3A). The cultures were performed at multiple cell concentrations as our laboratory has previously reported that cell density inversely correlates with IgE production (Rabah and Conrad, 2002), (Caven et al., 2005b). To determine physiological relevance, the studies were repeated with the natural ligand glutamate. A similar increase in the amount of IgE synthesized from purified human B cells in the presence of glutamate (Figure 3B) was seen. Thus, we chose to utilize the natural ligand for the remainder of the studies.

Our laboratory has previously shown that the addition of IL-21 can enhance IgE production in the human system (Caven et al., 2007) due to the fact that IL-21 augments plasma cell development in human *in vitro* cultures. Thus, IL-21 was added into our culture system to determine if the glutamate mediated effect on IgE synthesis was still seen even in the presence of the additional cytokine stimuli. This effect is illustrated by the fact that cultures with IL-21 and glutamate make much more IgE than either IL-21 alone or with glutamate alone as indicated by the scale on the y axis of the ELISA graphs (Figure 3C).

RPMI-1640, the primary media most utilized for lymphocyte culture, contains 0.1mM glutamate. To better control for and determine the actual role of the glutamate mediated increase in IgE, we switched these studies to using DMEM based media, which lacks any glutamate. In the DMEM based media we observed a pronounced increase in the amount of IgE generated in the presence of glutamate and IL-21(Figure 3C). Thus all remaining studies will employ the use of DMEM based media with the addition of IL-21.

To confirm that the glutamate mediated increase in IgE is mediated through the kainite receptor, the antagonists used in the soluble CD23 studies were employed. As evidenced in Figure 3D, we see no effect with either vehicle (DMSO) or the AMPA antagonist, NBQX. However the KAR antagonist, NS102, almost completely blocks the glutamate mediated increase in IgE production. While the IgE production in wells containing both Glu and NS-102 is still significantly higher than the control wells lacking Glu the amount of IgE produced is dramatically reduced ($p<.00001$) when compared to Glu containing cultures. In separate experiments, TPM also did not influence IgE production (data not shown), further confirming the KAR specificity.

KAR Activation leads to an increase in cell proliferation

Hasbold et al. (Hasbold et al., 1998) reported that cell proliferation is directly related to Ig production, thus B cells were cultured in the presence or absence of Glu to examine changes in proliferation. Our laboratory has shown that B cell proliferation and human IgE synthesis are correlated and increased in the presence of IL-21, thus extending upon the Hasbold study (Caven et al., 2005a). In order to examine if this phenomenon held true in the presence of KAR agonism, primary human B cells were grown in the presence/absence of glutamate and cell proliferation was determined. From the data shown, KAR activation leads to a significant increase in proliferation (Figure 4) which is prevented in the presence of NS102. Taken together, our data coupled with previous data shown by other groups indicating the need for a proliferative response required for IgE synthesis clearly strengthens the argument that KAR activation via glutamate signaling can promote an enhanced humoral response and the enhanced IgE production would be anticipated to enhance an atopic phenotype.

KAR activation increases IgG synthesis

To determine if glutamate stimulation through the KAR would enhance other immunoglobulin isotypes, we examined total IgG and IgM secretion from B cell cultures. A similar phenomenon in terms of IgG production was observed as compared to the glutamate mediated IgE enhancement. Enhancement in IgG production was seen in all but the highest density cultures (Figure 5A). This enhancement was blocked by the KAR antagonist, NS102, but was unaffected by either vehicle control or the AMPA antagonist, NBQX (Figure 5B). However, total IgM levels were not influenced. The IgM data is shown at a single concentration (150,000 cells/well) but no enhancement of IgM was seen at any cell concentration (Figure 5C).

Glutamate enhancement is also seen in PBMC cultures

The aforementioned studies used purified B cells. In order to determine if the enhancement was still effective with the cellular milieu that closely mimics the immune system, we examined effect of KAR agonists on IgE production using PBMC. As can be seen in Figure 6A, addition of glutamate to the PBMC cultures that are stimulated with anti-CD40, IL-4, and IL-21 again resulted in a very strong enhancement of IgE synthesis. Other immunoglobulins (total IgG and IgM) were also tested and while IgG enhancement was seen (Figure 6B), IgM production in the PBMC cultures, as with purified B cells, was not significantly influenced (Figure 6C). The KAR specific antagonist, NS-102, again strongly blocked the increase in IgE and IgG production. Note that the cell concentrations used for the NS102 were where maximum Ig production was seen. In a similar fashion as with purified B cells, proliferation was also affected by the addition of glutamate (Figure 6D).

Glutamate enhancement of sCD23 and Ig production is ADAM10 Dependent

Ortiz *et al.* originally reported that KAR activation increased ADAMs other than ADAM10 and KAR activation has been linked to the increase in several other matrix

metalloproteinases (Szklarczyk et al., 2002; Flood et al., 2007). Hence we next wanted to determine if the KAR mediated increase in sCD23 was due to ADAM10 activation. Prior to the addition of glutamate, RPMI8866 were incubated with 10μM ADAM10 specific inhibitor. Figure 7A shows that glutamate cannot overcome the ADAM10 mediated inhibition of sCD23 further indicating that the increase in sCD23 observed in the presence of glutamate is mediated through a KAR specific activation of ADAM10.

While ADAM10 is appreciated to be the CD23 sheddase, the effects of ADAM10 on immunoglobulin production have not been directly examined. Primary human B cells were treated with an inhibitor of ADAM10 to determine the effects of ADAM10 inhibition on human immunoglobulin production. From the data presented in Figure 7B, it is evident that ADAM10 inhibition blocks CD23 shedding as expected as well as IgE and IgG production; however no effect was seen on IgM. Interestingly this data inversely correlates with our KAR studies in that ADAM10 increases lead to an increase in sCD23, IgE, and IgG with no effect on IgM. Thus we can show that ADAM10 modulation does indeed impact B cell Ig production.

Discussion

This study is one of the first to show that a functional glutamate receptor of the kainite subtype exists in the human immune system. While there are studies to date which report that glutamate receptors exist on human T cells (Ganor et al., 2003a) and macrophages (Boldyrev et al., 2004), their presence has not been demonstrated on B cells. While those studies are important in illustrating that glutamate receptors do exist in the immune system, our study is pivotal in that receptors of the kainate subtype to our knowledge have never been reported to exist in lymphocytes. The only other KAR demonstration in the hematopoietic system was the very recent finding that KARs are on platelets, and stimulation thereof promoted cyclooxygenase activation (Sun et al., 2009).

Aside from the presence of the receptors themselves functional relevance was also shown. We focused on ADAM10 as our laboratory has recently identified this protease to be responsible for the cleavage of CD23 from the cell surface (Weskamp et al., 2006). In view of the recent demonstration that ADAM10 is the primary CD23 sheddase, we searched for agents that would modify ADAM10 activity. The overall purpose was to test the hypothesis that ADAM10 modulation would, by virtue being the CD23 sheddase, result in IgE modulation. It has been shown that specific ligation of the kainate receptor leads to an increase in ADAM10 mRNA in the CNS. Based upon the work by Ortiz *et al.* we wanted to determine if this kainite receptor existed in the immune system and activation of this receptor would lead to an increased expression of ADAM10 in a similar fashion in the immune system (Ortiz et al., 2005). This finding would allow for manipulation of ADAM10 as a direct means to influence B cell biology in the human system. While the Ortiz paper also illustrates that ADAMs 9 and 15 are also up-regulated in the presence of KA, no involvement of these two ADAMs in CD23 ectodomain shedding events was found. Normal CD23 cleavage was found using both ADAM9 and ADAM15KO mice, thus, up-regulation of ADAM10 is responsible for the changes in the CD23 observed (Weskamp et al. 2006). Furthermore, the use of the ADAM10 specific inhibitors confirms both the importance of ADAM10 in B cell immunoglobulin production as well as specific actions of KAR activation leading to ADAM10 activation.

In addition to its effect on CD23 shedding, KAR activation also leads to a significant increase in cell proliferation. As evidenced by our group as well as others, cellular proliferation is a key element in generating strong class switch recombination as multiple rounds of cell divisions are needed for this to occur (Hasbold et al., 1998). In the human

system, it is known that at least eight cell divisions are needed in order to produce IgE (Caven et al., 2005a). Thus, the increase in proliferation may help explain the increased class switching observed as indicated by elevated levels of total IgG and IgE. Note that class switching occurs both in the presence and absence of KAR stimulation and that what is seen is a dramatic increase in the synthesis of class switched Ig. There is precedent for KAR activation to cause increasing cell proliferation as it has been shown in the literature that elevated glutamate levels promote growth of human histiocytic lymphoma cells (Haas et al., 2005), yet the mechanism for this increase was not examined. Increased CD23 cleavage also correlates with elevated IgE production and this may also relate to the results shown here as past studies have highlighted CD23's important role as a negative regulator of IgE synthesis. Given the relatively modest influence on cell divisions (Figure 4), enhancement in proliferation is not likely to be the complete explanation for the Ig production enhancement. The increased ADAM10 activity results in increased sCD23. This has been shown to correlate with increased IgE production in the mouse system (Ford et al., 2006) as well as the human (Saxon et al., 1990). High membrane CD23 levels, caused by transgene over-expression (Payet-Jamroz et al., 2001) resulted in decreased IgE and IgG1 expression, at least in the mouse system. Thus, CD23 alteration may at least provide a partial explanation for the results shown in this study, but the mechanism will require additional studies which will examine intracellular events post KAR activation. Such studies are currently underway in our laboratory.

Interestingly, the receptor described by Ortiz was a glutamate receptor whose expression has primarily been reported to be CNS specific. As aforementioned, elevated glutamate levels have recently been implicated in a variety of peripheral diseases such as certain cancers, HIV, epilepsy, RA, and SLE and certain treatments for autoimmune disease can also affect glutamate levels. It is remarkable to note that many of these diseases also have an accompanying increase in immunoglobulin production. For example, elevated serum IgE and increased prevalence of atopy is reported in patients infected with HIV (Bowser et al., 2007). Frediani et al. (Frediani et al., 2001) performed a cohort study with 72 pediatric patients with epilepsy compared to 202 healthy age matched controls. This study showed a significantly increased incidence of allergies in the epileptic population as opposed to the control. One hallmark of B cell mediated autoimmunity is that patients have an accumulation of auto-reactive B cells which are hyper proliferative and produce pathogenic immunoglobulin, typically of the IgG isotype. In the case of SLE, other documented, but perhaps lesser known attributes of the disease include elevated levels of IgE (Atta et al., 2004) and elevated levels of soluble CD23 (Bansal et al., 1992). Elevated levels of sCD23 have also been reported in rheumatoid arthritis and Sjogren's syndrome (Bansal et al., 1994; Bansal et al., 1992). The aforementioned phenomena all clinically correlate to the phenomena we observe *in vitro* in the presence in KAR activation, suggesting that glutamate enhancement of IgG and IgE production may play a role in the disease progression.

One may question that the concentration of glutamate used in our studies may not be physiologically relevant, however it is reported that glutamate concentration in the brain are as high as 15mM (Danbolt, 2001). In the periphery serum glutamate levels may not reach levels as high as these but they have been reported in the millimolar range (Lyoo et al., 2009; Alstergren et al., 2010). Furthermore, it is appreciated that dendritic cells release glutamate themselves and directly act on T cells in the immunological synapse (Affaticati et al., 2010; Pacheco et al., 2010). Thus it is very feasible that the localized concentration of glutamate released to the B cell would be in the ranges used in this study.

There continues to be mounting evidence indicating interplay between the immune and nervous systems such as the fact that cholinergic compounds are immunosuppressive (Wrona, 2006) and the fact that norepinephrine can stimulate the immune system (Nance

and Sanders, 2007). Despite evidence for these and other neurotransmitters, little is known about the effects of glutamate on the immune system and what is known focuses primarily on T cells and macrophages. With more and more evidence pointing to a “neuro-immuno dichotomy”, it is important to not only look at the immune system’s effects on the nervous system but vice versa as well. Taken together, this report serves to help elucidate the effects that a neurotransmitter signaling may exert on the immune system and may serve as a useful tool for developing new therapies for immune disease. Especially intriguing is the strong inhibition of Ig production seen with the KAR antagonist, NS-102. NS102 is an antagonist to the GluK2 containing KARs thus confirming specificity of the KAR subtype observed in the human immune system. As KAR knockouts showed minimal CNS effects (Mulle et al., 1998), NS-102 may represent a new tool to control Ig synthesis. Alternatively, it may be possible to produce related KAR antagonists that are engineered to not cross the blood brain barrier. These antagonists would potentially have no CNS issues and would thus have the potential to strongly reduce peripheral Ig production and control the IgG and IgE mediated diseases.

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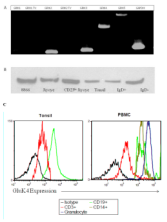

Figure 1. Kainate receptors are expressed in the human immune system

Human B cells were analyzed for the presence of kainate receptor subunits both at the RNA and protein level. (A) The human cell line RPMI8866 was examined by RT-PCR for the presence of multiple subunits of the kainate receptor. A representative agarose gel of three performed is shown. (B) Western blot shows presence GluK4 protein in multiple B cell sources. Shown is one representative Western out of three. (C) Flow cytometry of human leukocytes shows both presence of protein as well as cell surface expression of KARs based on GluK4 expression.

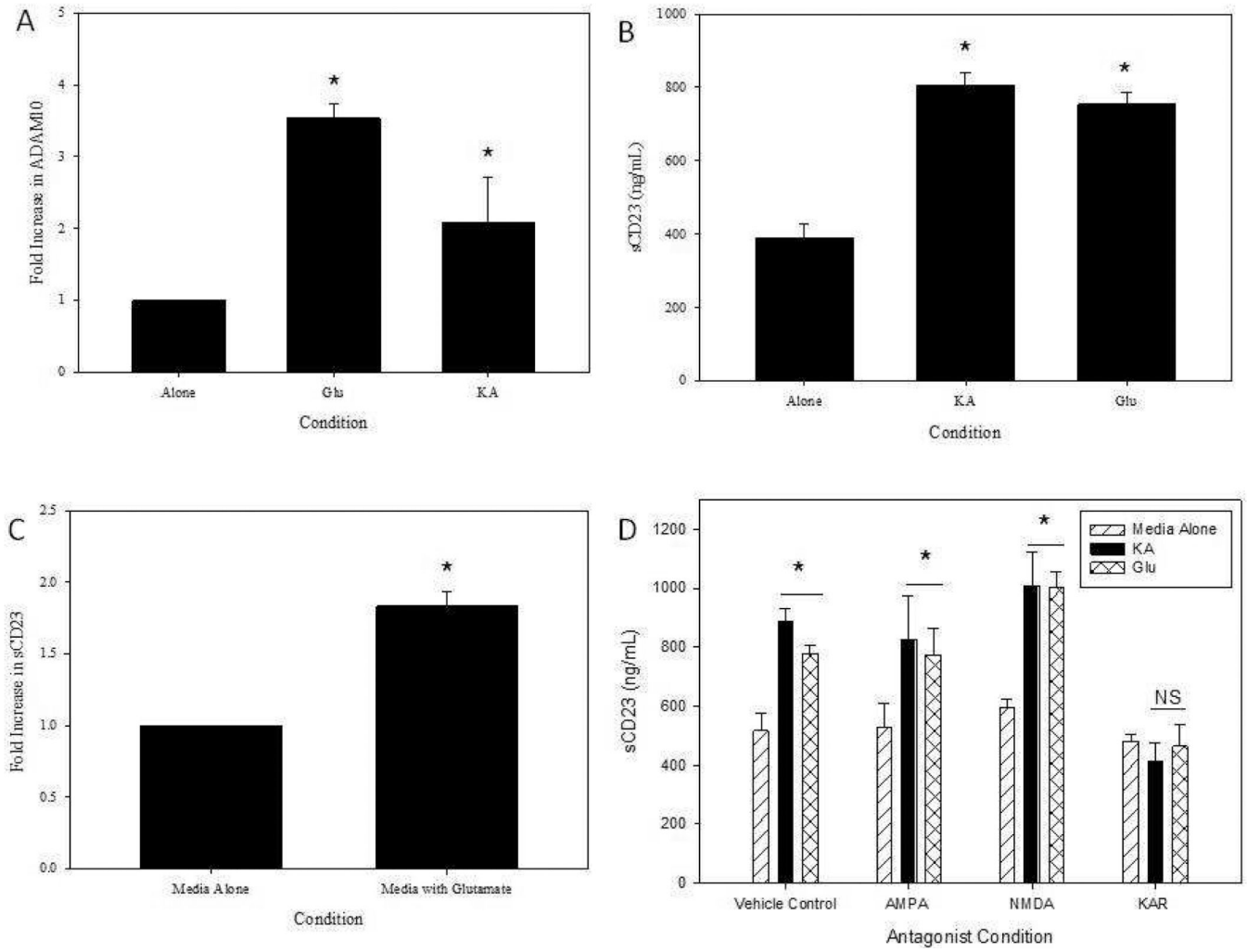

Figure 2. Kainate receptor activation increases ADAM10 levels

(A) Primary B cells were cultured in CDMEM-10 alone, 5mM glutamate, or 5mM kainic acid for 30 minutes. RNA was then isolated and subjected to qPCR analysis as described. Levels are normalized to human beta-actin. Shown is the average of three separate donors. (B) Soluble CD23 release is also increased post KAR activation in RPMI8866 cells and primary human B cells (C). Figure C is the sum of four individual donors so data is graphed as fold increase to normalize data to account for individual variance. (D) RPMI8866 cells were pretreated with either vehicle control (DMSO), a AMPAR antagonist (NBQX), a NMDAR antagonist (TPM), or a KAR antagonist (NS102) (50μM) for one hour prior to the addition of 5mM glutamate or 5mM KA. 24hours later, sCD23 levels were determined as described. Shown is the average +/− SE of three individual experiments. *Significant at p<. 05; NS – not significant

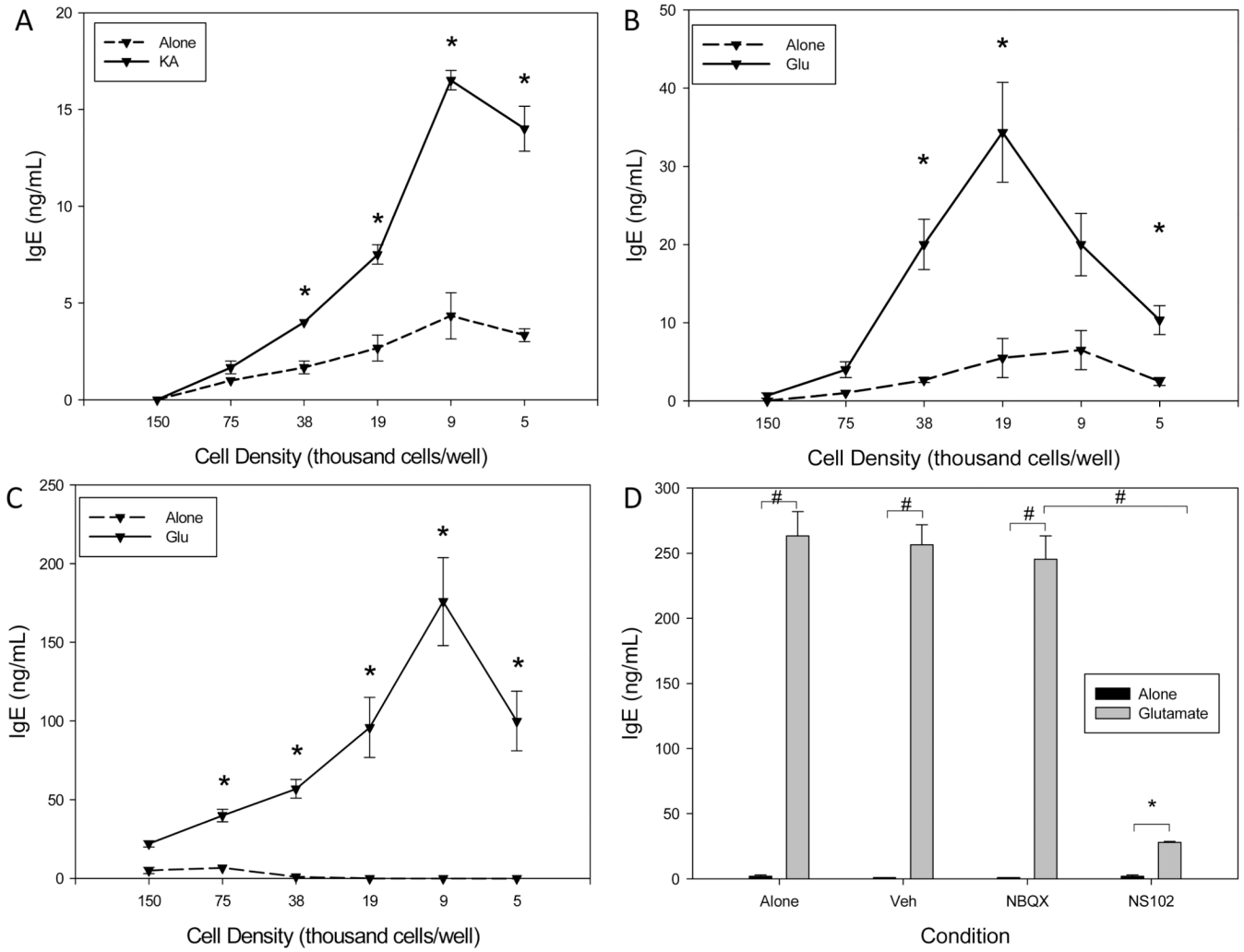

Figure 3. Kainate receptor activation increases IgE synthesis

Primary B cells were cultured in the presence of 10 ng/mL IL-4 and 1 μg/mL anti-CD40 in CRPMI-10 in the presence or absence of 5mM KA (A) or 5mM Glu (B). After 14 days of culture, cell free supernatants were analyzed via ELISA for IgE levels. (C)Primary B cells were cultured in the presence of IL-4 and anti-CD40 as in (A) plus 200ng/mL IL-21 in the presence or absence of 5mM glutamate in CDMEM-10. After 14 days of culture, cell free supernatants were analyzed via ELISA for IgE levels. (D) Before culture primary B cells were treated with 10μM vehicle (DMSO), NBQX, or NS102. Primary B cells were then cultured in similar conditions as in C. After 14 days, ELISA analysis performed. Part A, B, and C represent three different individuals that serve as a representative donor. Part D is the cell concentration in which maximum Ig production was observed (9,000 cells/well) either in the presence or absence of antagonist from another donor. Both the RPMI and DMEM culture experiments have been performed a minimum of three times with similar results. * indicated $p<0.05$ as compared to indicated control. # indicates $p<0.01$ as compared to indicated control.

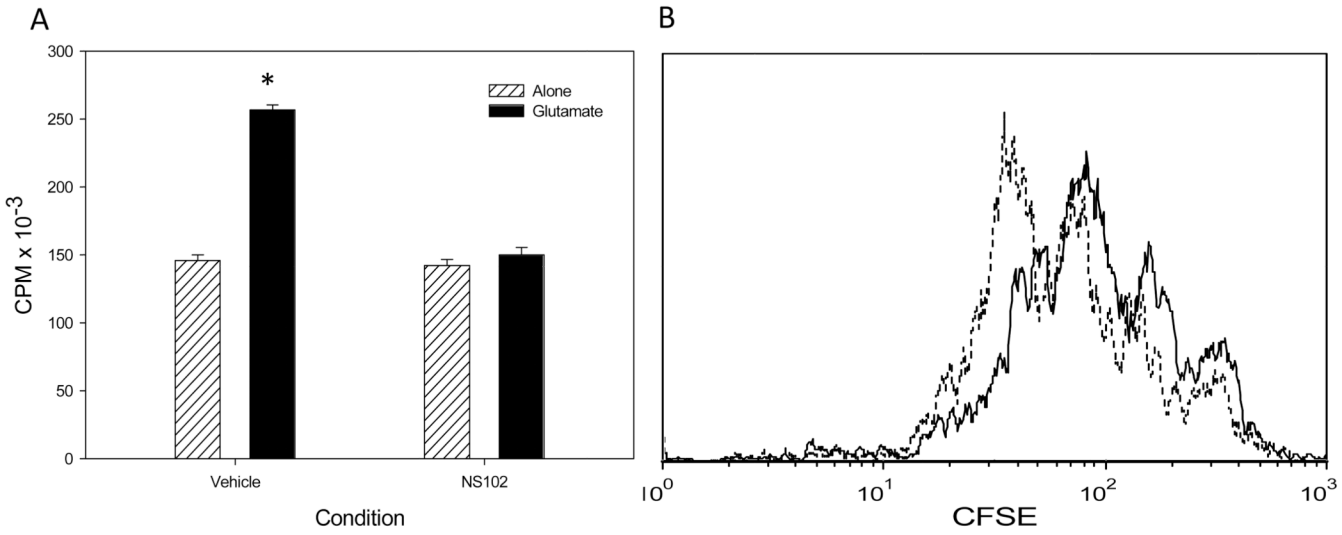

Figure 4. Kainate receptor activation increases cellular proliferation

(A) Primary B cells were cultured as in Figure 3C in CDMEM-10 in the presence or absence of 5mM glutamate at a concentration of 150,000 cells/well. Prior to the addition of glutamate, cells were pretreated for 1 hour with 10μM vehicle (DMSO) or NS102. After 96 hours, cells were pulsed for 24 hrs with [^{3}H]-thymidine. Shown is one representative donor of three performed. * indicates $p<0.05$ as compared to vehicle alone. (B) Primary B cells (10^4 /well) were labeled with CFSE as indicated in Materials and methods and were cultured with IL-4, anti-CD40, IL-21 in CDMEM-10 alone (——) or in the presence of 5mM glutamate (−·−·−).CFSE fluorescence was determined on day 5 post culture initiation. Part B representative of two of similar design.

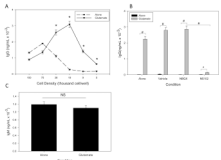

Figure 5. Kainate receptor activation increases total IgG synthesis

Primary B cells were cultured as in Figure 3C in CDMEM-10 in the presence or absence of 5mM glutamate. After 14 days of culture, cell free supernatants were analyzed via ELISA for IgG levels (A) or IgM levels (C). (B) Before culture primary B cells were treated with 10μM vehicle (DMSO), NBQX, or NS102. Primary B cells were then cultured in similar conditions as in (A). After 14 days, ELISA analysis performed. Shown in B is one cell concentration with highest Ig production (38,000 cells/well).Each panel is one representative donor. Experiment shown is one of three with similar outcomes. * indicated $p<0.05$ as compared to alone. # indicates $p<0.01$ as compared to indicated control. NS – not significant.

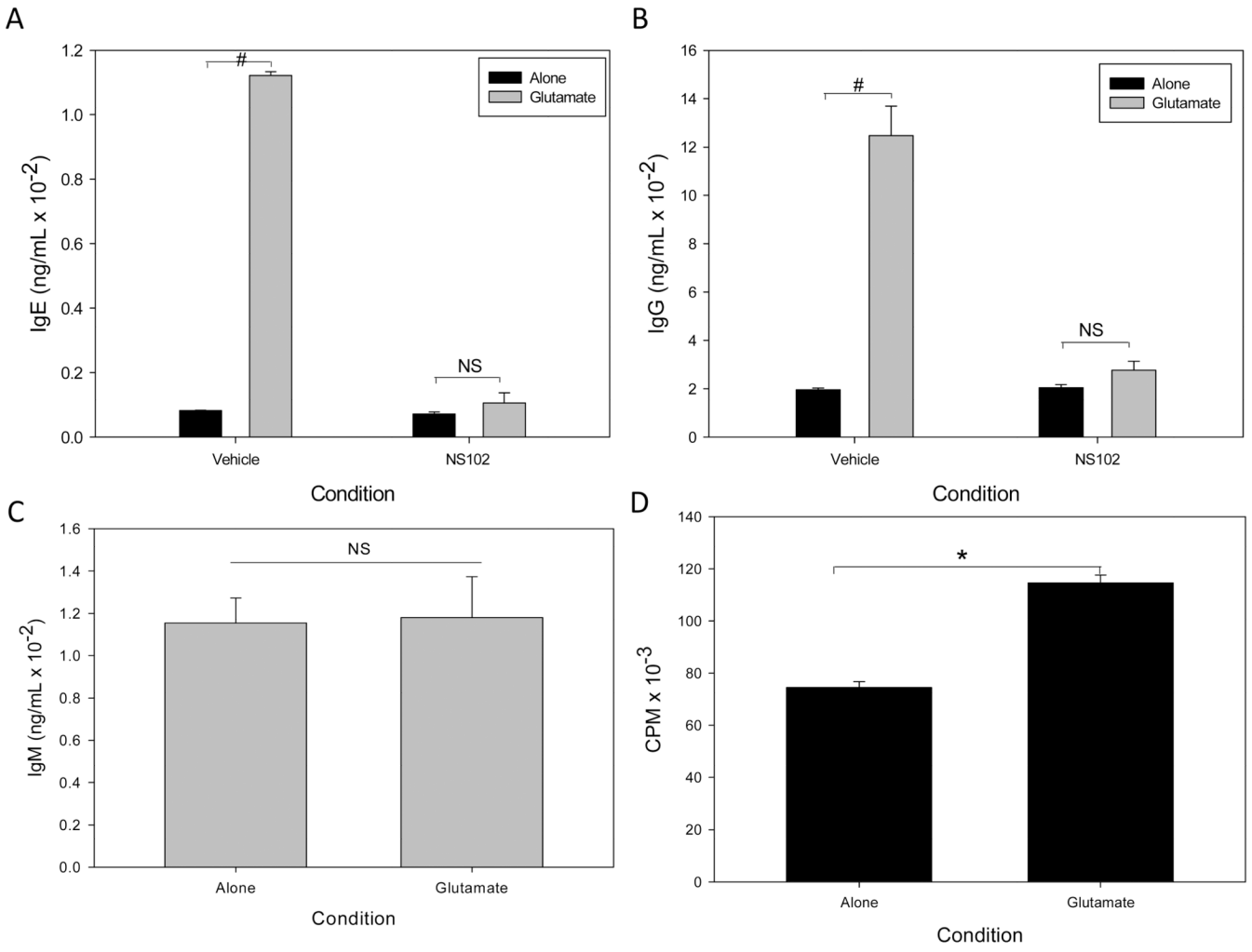

Figure 6. Kainate receptor activation in human PBMC model

Human PBMC were cultured in the presence of 10ng/mL IL-4, 1 μg/mL anti-CD40, and 200 ng/mL IL-21 in CDMEM-10. Prior to the addition of glutamate, cells were pretreated for 1 hour with 10μM vehicle (DMSO) or NS102. After 14 days of culture, cell free supernatants were analyzed for IgE (A), total IgG (B), or IgM (C). The cell concentration showing maximum Ig production is shown in the presence or absence of Glu. These cell concentration values were 18,000 cells/well (A), 38,000 cells/well (B) and 300,000 cells/well (C). Separate, identical cultures (150K/well) were plated to analyze cellular proliferation (D). Shown in each is one representative donor. A total of 3 PBMC cultures have been examined in this manner with similar results. * indicated $p<0.05$ as compared to alone. # indicates $p<0.01$ as compared to indicated control. NS – not significant.

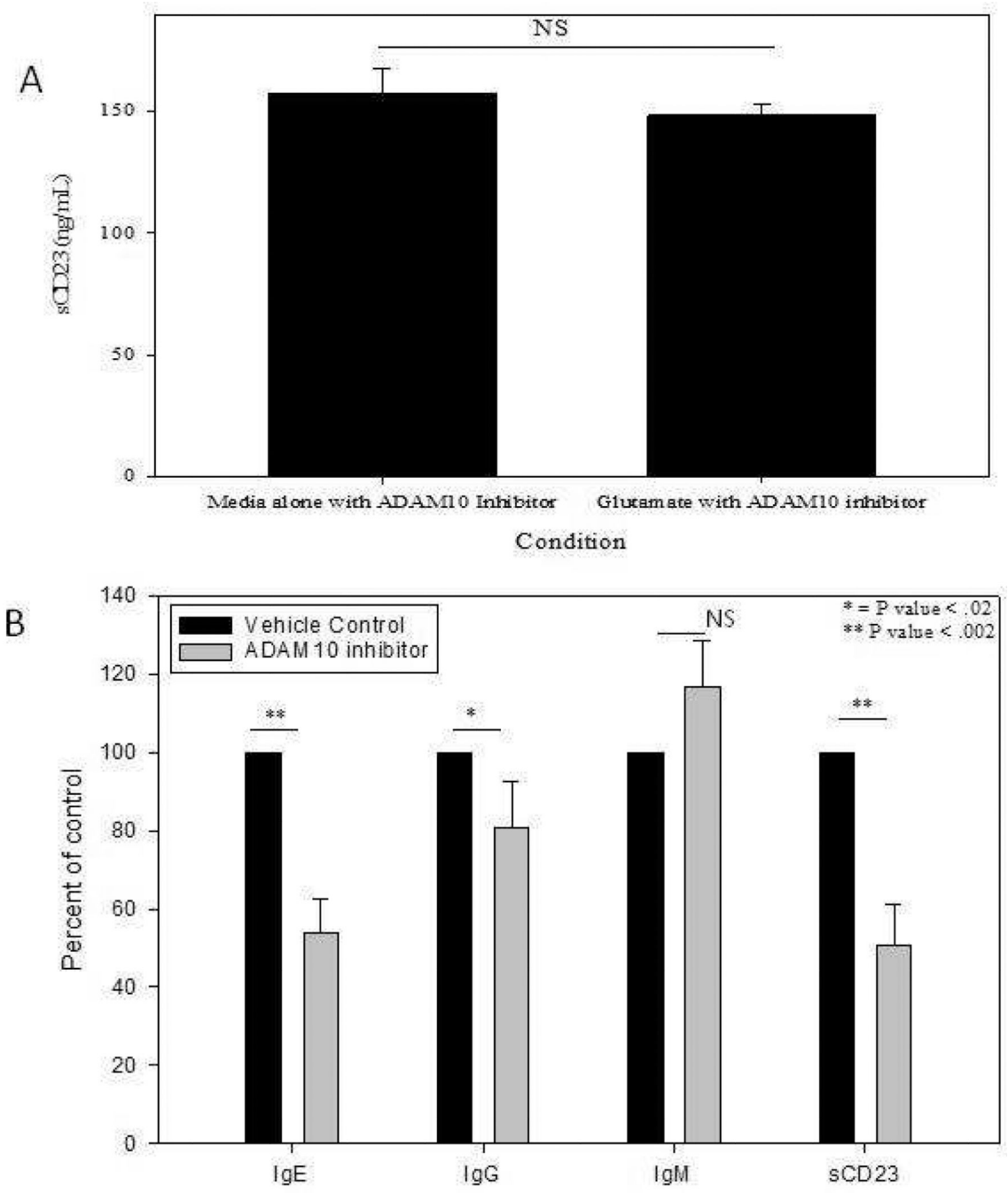

Figure 7. Glutamate enhancement of sCD23 and Ig production is ADAM10 Dependent

(A) RPMI8866 cells were treated with 10μM ADAM10 inhibitor or vehicle control prior to the addition of 5mM glutamate. 24 hours later cell free supernatants were harvested for sCD23 release. (B) Primary human B cells were cultured with 10ng/mL IL-4, 1μg/mL a-CD40, and 200ng/mL IL-21 in the presence of 10μM ADAM10 inhibitor or vehicle control. Cell free supernatants were analyzed for either sCD23 on day 5 or Ig production by ELISA on day 14 as described. Shown is data summary for five donors. Data is normalized and shown as percent inhibition.