

Interleukin-6 (IL-6) may be considered the prototypic multifunctional cytokine. Indeed, this is reflected in the many names assigned to it before its final designation in 1987 [1]. Human IL-6 is a 26kDa glycoprotein produced by several cell types including macrophages and lymphocytes. Macrophages, with T and B lymphocytes, and dendritic cells comprise an immune compartment that in joints of patients with rheumatoid arthritis (RA) replaces what was the subintimal layer of synovium [2]. IL-6 is able to degrade cartilage and erode bone [3], and it is responsible for the production of acute phase reactants such as C-reactive protein [4].

In patients with RA, concentrations of IL-6 in serum correlate with disease activity and extent of joint damage, and reduction in disease activity after treatment with disease-modifying antirheumatic drugs (DMARDs) is associated with reductions in serum concentrations of IL-6 [5]. Several small clinical trials suggest efficacy of treatment of RA patients with antibody to IL-6 or its receptor [6]. In addition, IL-6 induces spinal cord injury in patients with immune mediated transverse myelitis [7], and it has been implicated in the immunopathogenesis of systemic lupus erythematosus [8]. Thus, inhibition of IL-6 production may be a useful strategy for treatment of patients with RA and other immune mediated diseases characterized by inflammation and tissue injury.

The *Cannabis* plant has been a source of medicinal preparations since the earliest written records on pharmacobotany [9]. A major obstacle to broad acceptance of cannabinoids as therapeutic agents is their psychoactive effects. A class of cannabinoids, the carboxyl tetrahydrocannabinols, which are metabolites

of tetrahydrocannabinol (THC), shows promise as therapeutic agents that are free of cannabimimetic central nervous system activity [10]. These compounds, called cannabinoid acids, include all the carboxylic acid metabolites of the cannabinoids and their synthetic analogs. One analog, 1'1'-dimethylheptyl-THC-11-oic acid, termed ajulemic acid (AjA), is a potent antiinflammatory and analgesic agent in several animal models [11, 12]. In addition, AjA is not psychoactive in mice. In fact, AjA suppresses THC-induced catalepsy in mice (10). Moreover, administration of 80 mg/day AjA for 1 week to patients with neuropathic pain relieved symptoms and did not induce behavioral changes [13].

Oral administration of AjA to rats with adjuvant arthritis prevents joint tissue injury in this animal model [14]. AjA binds to and activates the nuclear receptor peroxisome proliferator activated receptor gamma (PPAR γ) *in vitro* [15]. Therefore, we investigated the influence of AjA on IL-6 production by and release from human monocyte derived macrophages (MDM). We also examined the role of PPAR γ in the mechanism of action of AjA.

MATERIALS AND METHODS

Reagents. AjA was obtained from Organix (Woburn, MA). Its purity was monitored on high-pressure liquid chromatography by comparison with material synthesized previously [16]. The sample was 97% chemically pure, and was >99% chirally pure in the R, R enantiomer. AjA was dissolved in DMSO, then diluted with minimal essential medium (MEM) 2% fetal bovine serum (FBS) to

achieve appropriate concentrations. The concentration of DMSO was kept constant at 0.3%. GW9662 and troglitazone were from Biomol Laboratories Inc.

Establishment of Monocyte Derived Macrophages (MDM). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation in the usual manner [17]. PBMC (10^7 /mL) in RPMI-2% FBS were incubated overnight at 5% CO₂ and 37°C in 6 or 12 well tissue culture plates. Non-adherent cells were removed and media was replaced with RPMI containing 10%-15% FBS. Cultures were maintained for 4-6 days in a 5% CO₂ humidified incubator at 37°C. Media was then replaced with RPMI-2% FBS or other defined media according to experimental protocol. Cells were exposed or not to AjA (3-30µM) for 60 min., then stimulated with 10ng/ml LPS. Cells were collected at 4hr for assessment of IL-6 gene expression, and supernatants were collected at 18-24hr for measurement of secreted IL-6.

Measurement of IL-6 mRNA by the Hybridization/Colorimetric Assay. Quantikine mRNA (R&D Systems) is a colorimetric microplate method used to quantify cytokine-specific mRNA at low levels [18]. RNA samples were hybridized with mRNA-specific biotin-labeled “capture” probes in a microtiter plate. After the hybridization reaction was complete, samples were transferred to a streptavidin coated microtiter plate, and the RNA/probe complex was captured. Unbound material was washed away, and anti-digoxigenin alkaline phosphatase conjugate was added. Unbound conjugate was washed away, and substrate solution was added, followed by the addition of an amplification solution. Development of color is in proportion to the amount of IL-6 mRNA in the original sample. The reaction

was stopped, and the color intensity was measured with a standard plate reader at 490nm with background correction at 650nm. The minimal detectable level was 1.6amol/mL. Conditions were run in triplicate.

Measurement of IL-6 Protein. Supernatants were collected and analyzed for IL-6 by ELISA (R&D). Standards and diluted samples were incubated for 2hr at room temperature and then washed. Substrate solution was added to the samples for 30 min. Samples were then read on a microplate reader at 450nm with background correction at 540nm. The minimal detectable level was 3.1pg/ml. Conditions were run in triplicate.

Viability of Cells. The integrity of the MDM at the end of experiments was assessed by exclusion of trypan blue. Cells were diluted in 0.5% trypan blue. Nonviable cells lost their ability to exclude trypan blue, and stained blue. In no instance did the proportion of nonviable cells exceed 5%.

Statistical Analysis. Data were analyzed and compared by paired Student's test or by Student's *t*-test difference of means of independent groups.

RESULTS AND DISCUSSION

Release of IL-6 from MDM stimulated with LPS *in vitro* was suppressed by AjA in a dose dependent manner (Figure 1). In a series of 5 experiments, IL-6 secretion was reduced $69.1 \pm 6.0\%$ (mean \pm sd) by 10 μ M AjA ($p=0.001$ vs control cells not exposed to AjA). Similarly, reduction by AjA of IL-6 gene expression was significant and dose dependent (Figure 2).

We then tested the possibility of PPAR γ dependence by exposure of human MDM to the irreversible PPAR γ antagonist GW9662. AjA suppressed IL-6 release from LPS stimulated cells whether or not PPAR γ activity was blocked by GW9662. In addition, the known PPAR γ activator troglitazone [19] did not suppress IL-6 release, and suppression of IL-6 release by AjA was not blocked by GW9662 (Table 1). The results suggest that the action of AjA on IL-6 secretion from human MDM is PPAR γ independent.

Blockade of inflammatory cytokines such as tumor necrosis factor- α and interleukin 1 β (IL-1 β) is an effective strategy for the treatment of RA [20]. Interleukin-6 is a cytokine which can help maintain synovial inflammation and facilitate bone erosion [21, 22], and blockade of IL-6 action appears to be beneficial in treatment of patients with active RA [6]. In addition, a functional IL-6 gene is necessary for development of type II collagen induced arthritis in mice [23], and blockade of IL-6 prevents establishment of antigen-induced arthritis in mice [24].

Results of experiments presented here indicate that addition of AjA to human MDM *in vitro* reduces steady state levels of IL-6 mRNA and the subsequent secretion of IL-6 from activated cells. Similar results were obtained with human PBMC and synovial fibroblasts (not shown). However, the influence of AjA on MDM IL-6 was much more consistent, perhaps because of the greater homogeneity of the cell system. Experiments done with MDM in which PPAR γ activity was blocked indicate that suppression of IL-6 by AjA does not depend on PPAR γ activation. In addition, the known selective PPAR γ agonist, troglitazone,

does not suppress IL-6 release. Other PPAR γ ligands such as prostaglandin J₂ (PGJ₂) can also affect cell function in a PPAR γ independent manner [25].

PPARs were first cloned as nuclear receptors that mediate the effects on gene transcription of synthetic compounds called peroxisome proliferators.

Upregulation of PPAR γ reduces expression of several mediators of inflammation including IL-6 [26]. However, responses of cells to PPAR ligands can be due to activation of PPAR or can be PPAR independent [27] actions, which appear to be cell and stimulus, and perhaps, ligand specific. It is not unlikely that PPAR γ activation by AjA contributes to the therapeutic effect of the cannabinoid.

However, it appears from the studies presented here that the anti IL-6 action of AjA exhibited *in vitro* is not due to PPAR γ activation.

The precise mechanism whereby AjA suppresses IL-6 production by activated human MDM is not clear. Suppression by AjA of activation of the transcription factor NF κ B (Stebulis, et al, unpublished) might be important to suppression of inflammatory cytokines by AjA. T lymphocytes activated by IL-6 in the synovium of patients with RA can activate human monocyte/macrophages to induce expression of inflammatory cytokines and chemokines by NF κ B dependent and independent mechanisms [28]. It is of interest then, that AjA induces apoptosis of T cells [29], and suppresses activation of NF κ B (J. Stebulis, et al. unpublished). In addition, T lymphocytes in the joints of RA patients are resistant to apoptosis [30]. IL-6 rescues T cells from entering apoptosis *in vitro* [31], and suppression of T cell apoptosis *in vivo* leads to autoimmune arthritis in mice [32].

Inflammation is a well-orchestrated process designed to combat infection and prevent tissue injury. Like other cytokines, IL-6 can be defined as a modulating factor that balances initiation and resolution of inflammation. In diseases characterized by chronic inflammation, it appears that suppression of IL-6 prevents tissue injury [33]. It is likely that AjA acts *in vivo* on several targets to suppress immune mediated inflammation and tissue injury.

Of course, it is difficult to correlate results of experiments done *in vitro* with studies done *in vivo* in animal models. However, the evidence from animal studies [12] and experiments done *in vitro* which indicate that AjA suppresses monocyte IL-1 β production [17], enhances T cell apoptosis [29], and suppresses production of matrix metalloproteinases [34], and results presented here suggest that AjA may have value for the treatment of joint inflammation in patients with RA, osteoarthritis, and SLE. Successful therapy of joint tissue injury and of autoimmune disease will require modification of several aspects of host defense responses with agents that can be given safely for long periods of time. Nonpsychoactive cannabinoid acids meet those criteria.

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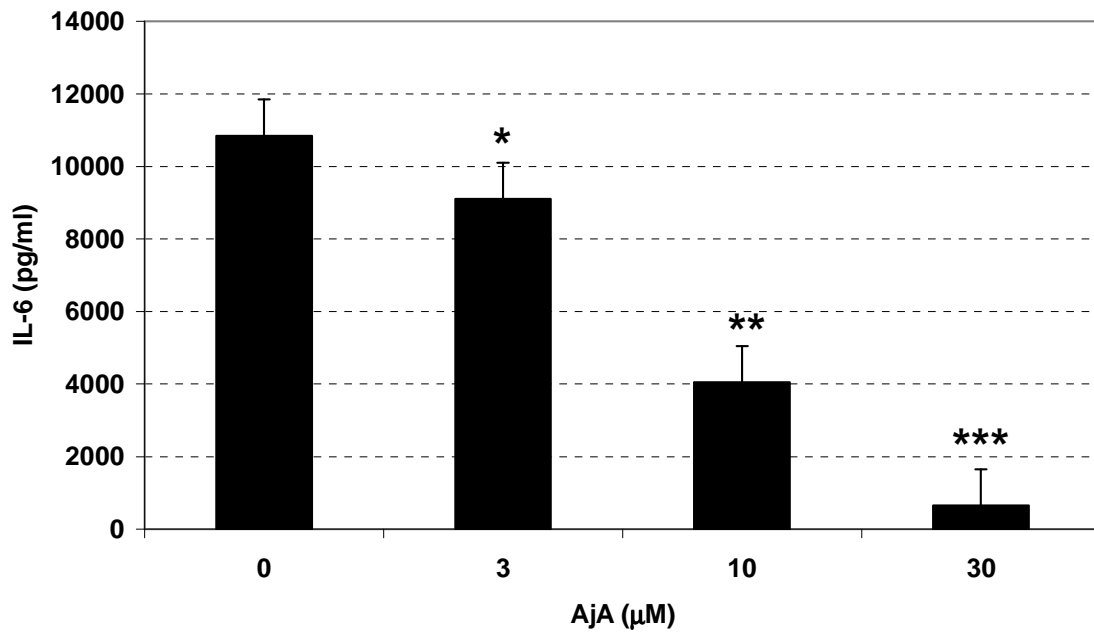


Figure 1. IL-6 release from human MDM. Cells exposed to AjA for 60 min, then stimulated 18hr with 10ng/ml LPS. IL-6 in supernatants measured by ELISA. Values are means of 3 experiments. All samples assayed in triplicate. Error bars are sd for 3 experiments. *p=0.03; **p=0.01, ***p=0.005, all vs. 0μM AjA.

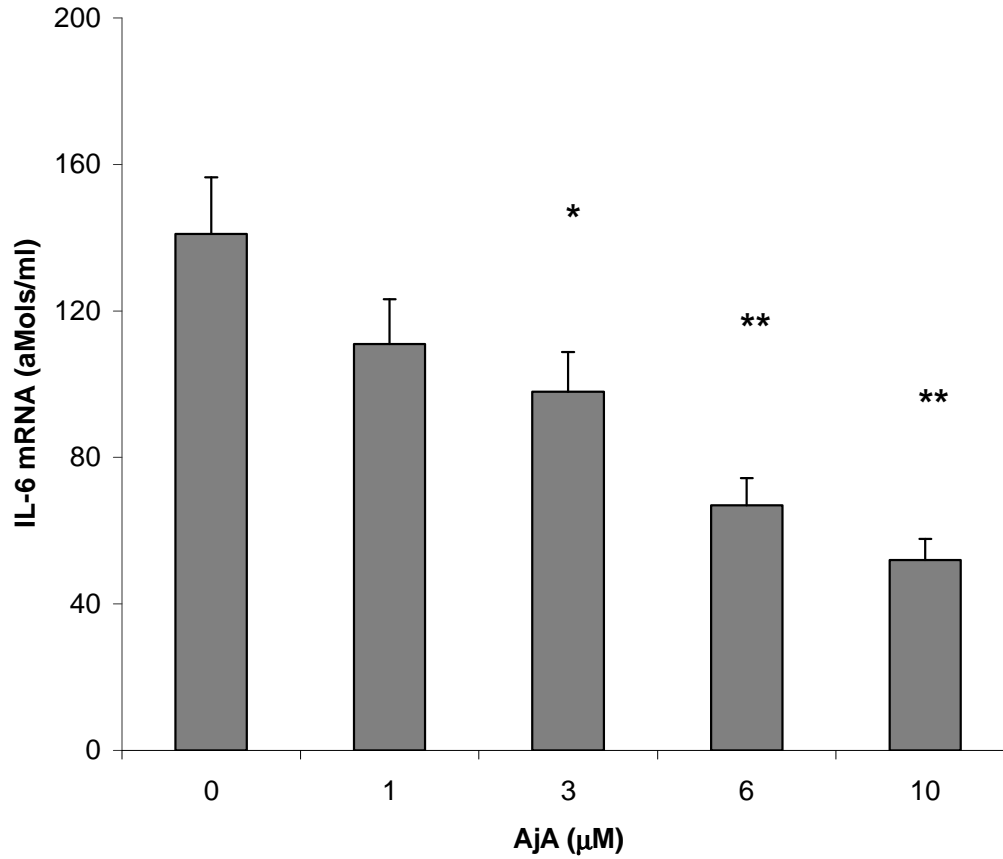


Figure 2. IL-6 gene expression in human MDM. Cells exposed to AjA for 60 min., then stimulated 4hr with 10ng/ml LPS. Steady state levels of cellular IL-6 mRNA measured by the hybridization/colorimetric assay. Error bars are sd for 3 experiments. * $p < 0.03$, ** $p = 0.02$, all vs. 0 μM AjA.

APPENDIX

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease predominantly affecting women of childbearing age. A common disease characteristic is that of continued remissions and relapses. At early stages, the disease can be difficult to diagnose, but hallmarks of the condition include antinuclear antibodies (98% of patients) and/or anti-double strand DNA (50% - 80% of patients) [36]. Arthritis is also common among patients with SLE.

Interferons are cytokines that were discovered to contribute to the resistance of viral infection. Their influence extends to cell proliferation, differentiation, survival of mature lymphocytes, and they have a central role in the immune response against infections [37]. Interferon-alpha (IFN- α) is involved in viral and innate immunity; and the adaptive immune response is mediated by interferon-gamma (IFN- γ) [38, 39]. IFN- γ has been found to play a key role in the development of both autoantibodies and nephritis in mice [40]. High levels of IFN- γ and IFN- α have been found in sera of some SLE patients [41] and levels of the cytokines correlate with disease activity [36, 42]. Thus, inhibition of pro-inflammatory cytokine production, specifically IL-6, IFN- γ and IFN- α , was investigated as a treatment strategy.

After showing that AjA (Figure A) can reduce IL-6 levels in normal volunteers, its effects on patients with SLE were studied. AjA has been shown to have a high potency with a lack of ulcerogenicity and a low dependence liability. Thus, it may be a good drug candidate for treatment of SLE by reducing the production of the aforementioned cytokines.

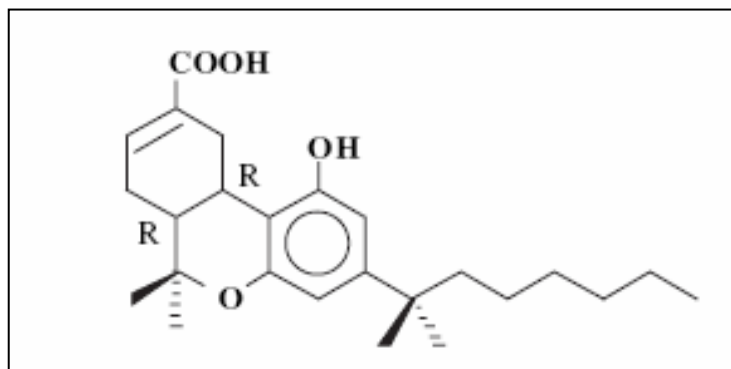


Figure A. Structure of Ajulemic Acid

Peripheral blood mononuclear cells (PBMC) were isolated from blood of SLE patients by Ficoll-Hypaque density gradient centrifugation and suspended in RPMI-2% FBS. The cells were separated into 6-well plates and incubated overnight at 5% CO₂ in a 37°C humidified incubator. For all experiments, cells were exposed to AjA for 60 min. For analysis of IL-6, IFN- α and IFN- γ levels in supernatants, PBMC were stimulated for 18hr with 10 μ M phytohaemagglutinin (PHA). Incubating for 4hr with Compound B (3M R848, TLR7, 20mM in DMSO) was used as an alternate stimulation method to experimentally determine IFN- α supernatant levels. IL-6, IFN- γ , and IFN- α levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA, R&D Systems).

Release of IL-6 from PBMC stimulated with PHA *in vitro* was suppressed by AjA in a dose dependent manner (Figure B). As shown in Figures C and D, stimulation of IFN- α by PHA and Compound B was not achieved. IFN- α levels were somewhat reduced, but without consistency. Similar to IL-6 results, levels of IFN- γ were also suppressed by AjA in a dose dependent manner (Figure E).

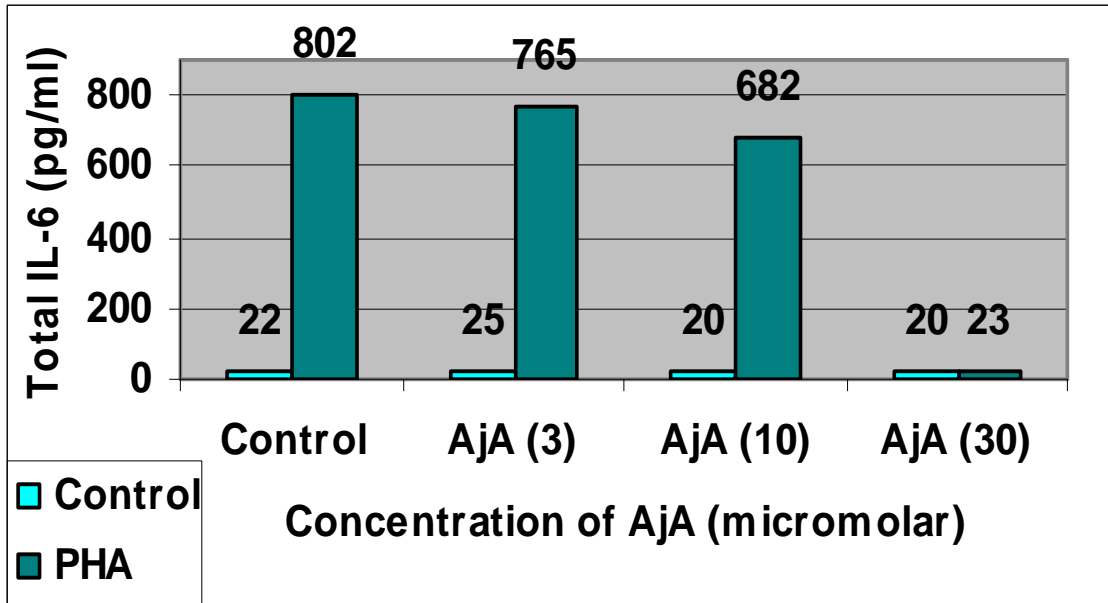


Figure B. IL-6 release from PBMC isolated from blood of SLE patient AB. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

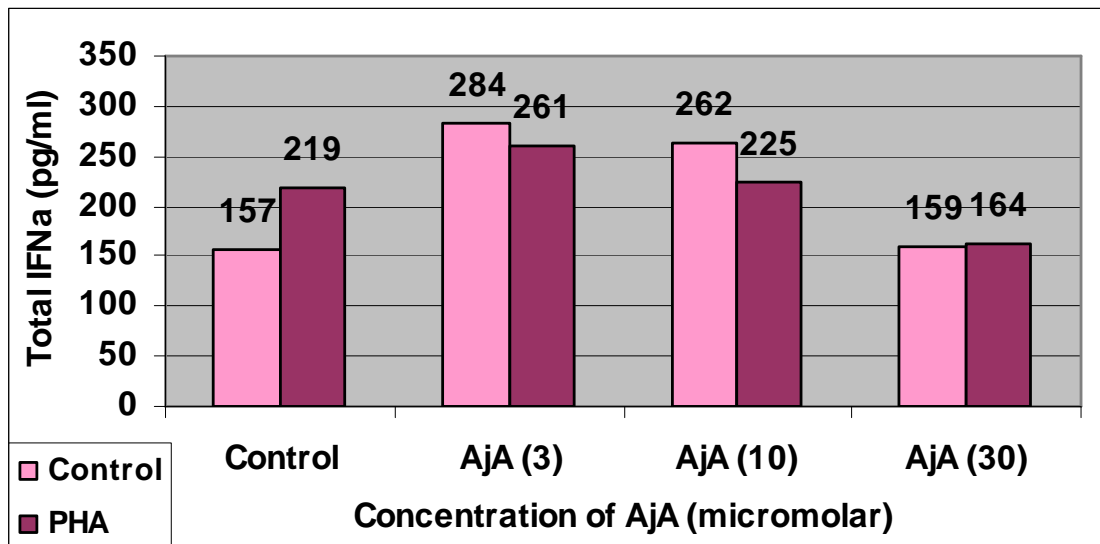


Figure C. IFN- α release from PBMC isolated from blood of SLE patient AB. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

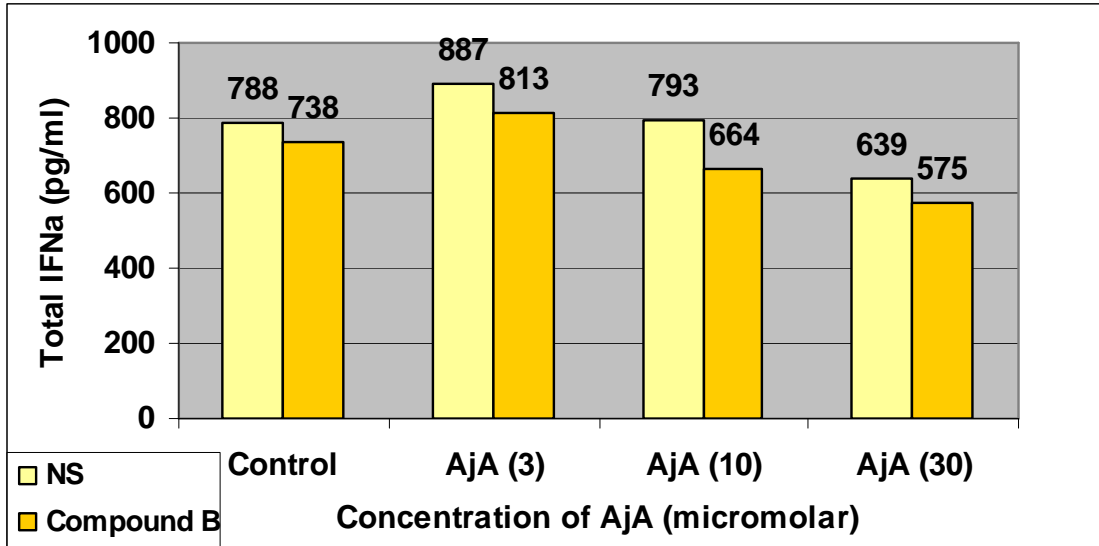


Figure D. IFN- α release from PBMC isolated from blood of SLE patient LP. Cells exposed to AjA for 60 min., then stimulated for 4hr with Compound B (1/250). IFN- α in supernatants measured by ELISA.

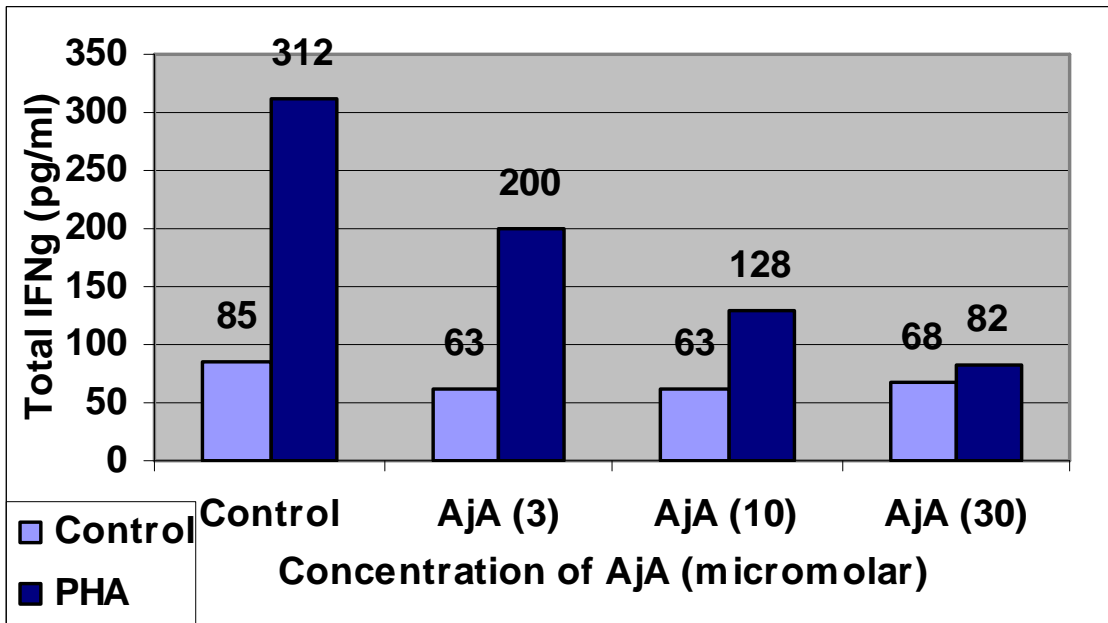


Figure E. IFN- γ release from PBMC isolated from blood of SLE patient AB. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

Figures B, C and E represent data from one experiment. The dose-dependent reduction shown in figures B and E was not observed in all samples tested. Common treatments for SLE such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, or anti-malarial medications [35] may have contributed to the variability of experimental results. If a patient is taking immunosuppressive medications, it is more difficult to obtain functional immune cells for study in culture. In addition, variations in levels of disease activity may also have affected the results of these experiments.

Ajulemic acid has the capacity to reduce levels of IL-6 and IFN- γ in a dose dependent manner. The reduction of these cytokines may correlate with a reduction in disease activity in SLE patients. The effects that AjA has on the interferon-inducible gene expression signature in patients with SLE should be investigated. Dysregulated expression of the genes in the IFN pathway has been observed in patients with SLE. Drugs that target this pathway may have considerable therapeutic benefits [38].

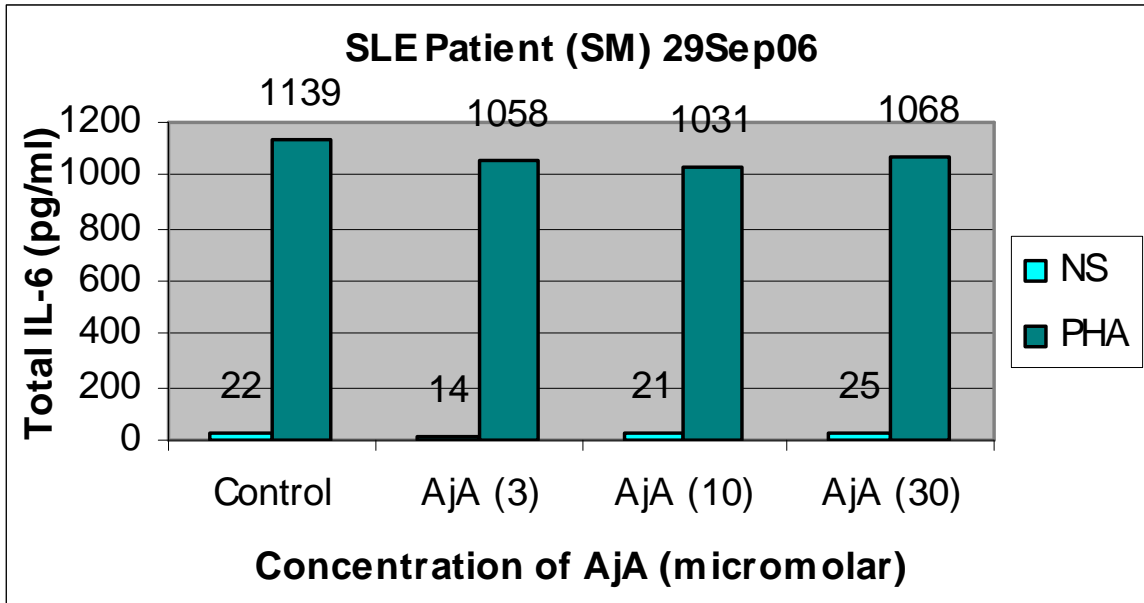


Figure B.1 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

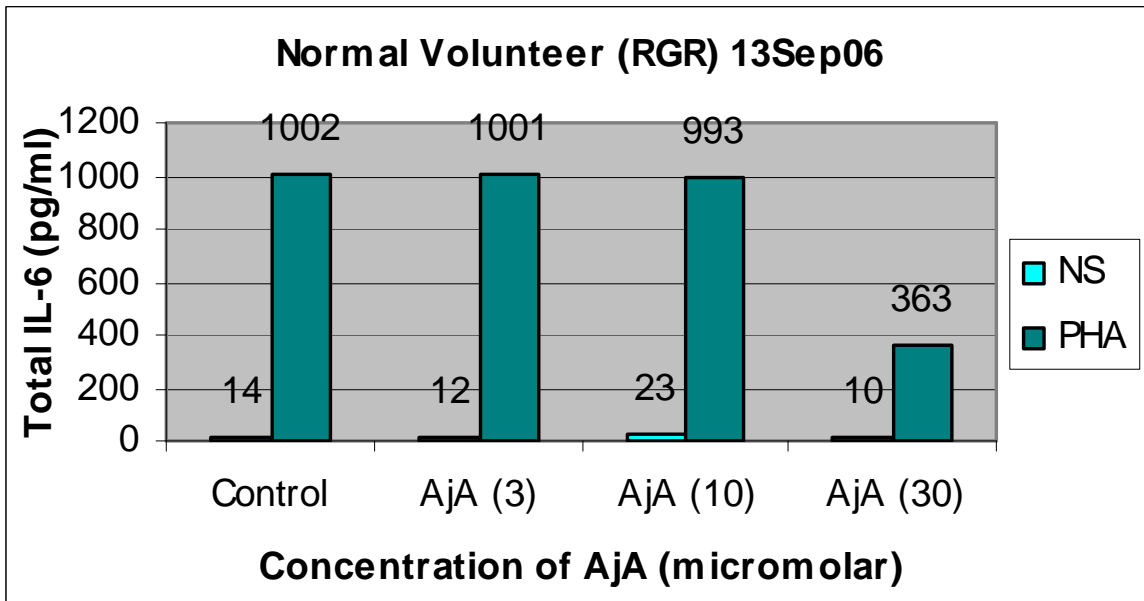


Figure B.2 IL-6 release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

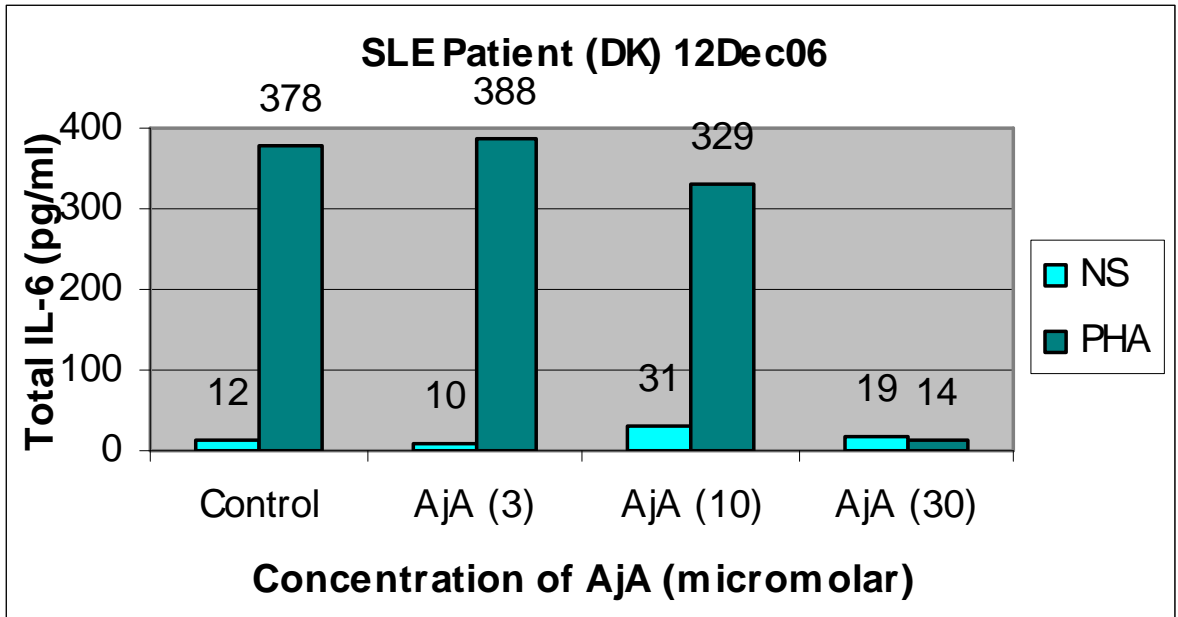


Figure B.3 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

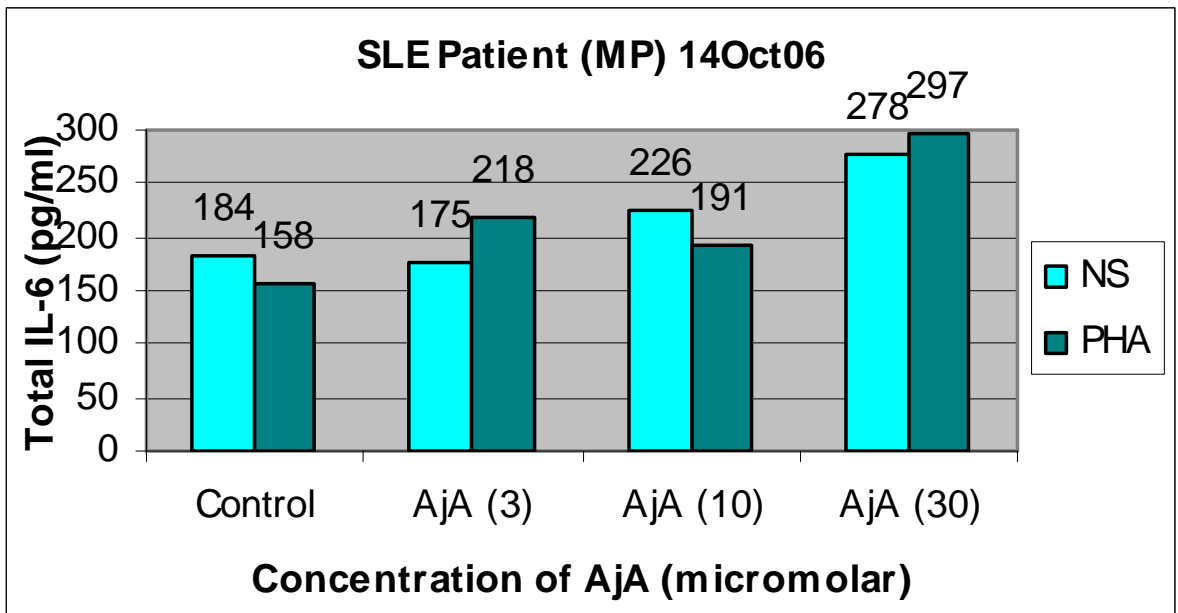


Figure B.4 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

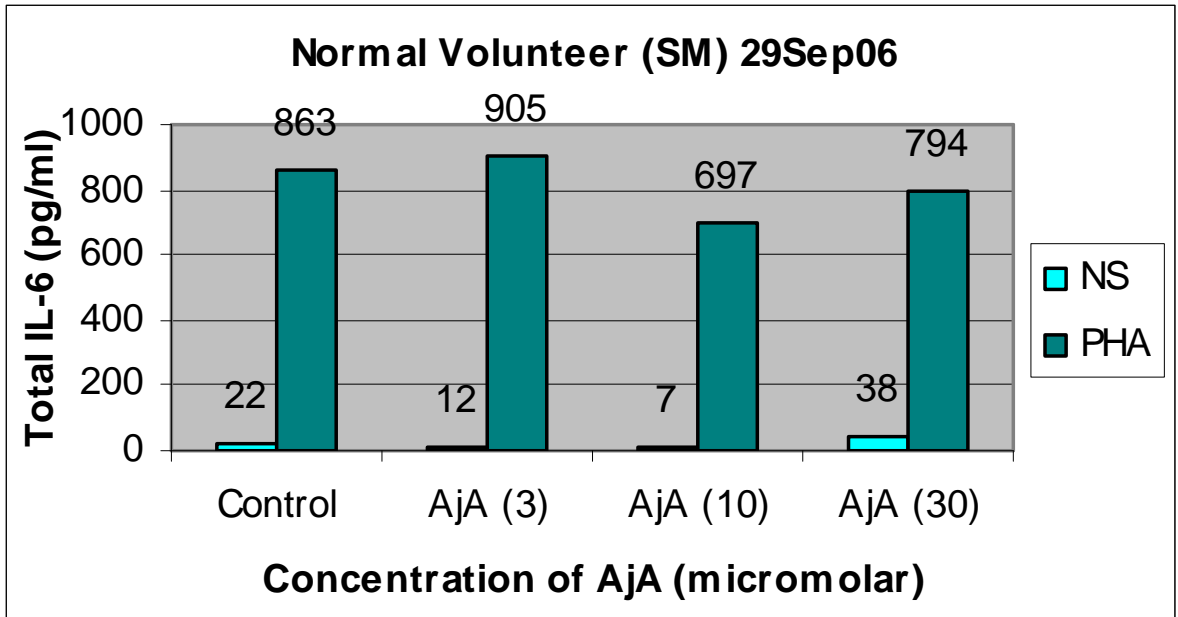


Figure B.5 IL-6 release from PBMC isolated from blood of a normal volunteer. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

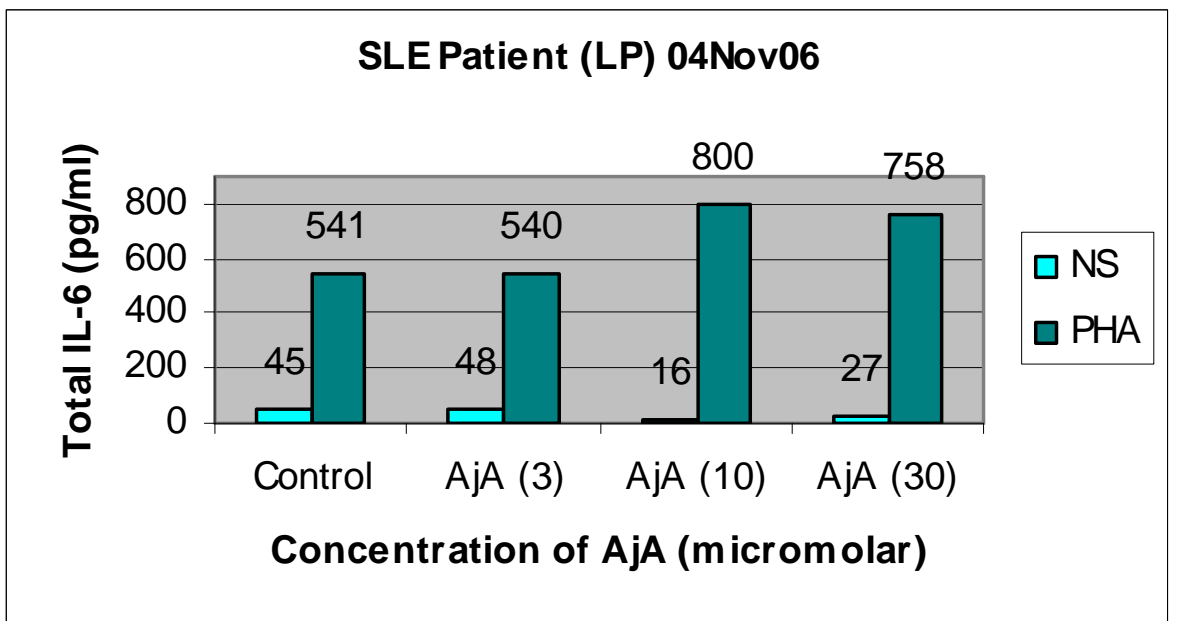


Figure B.6 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

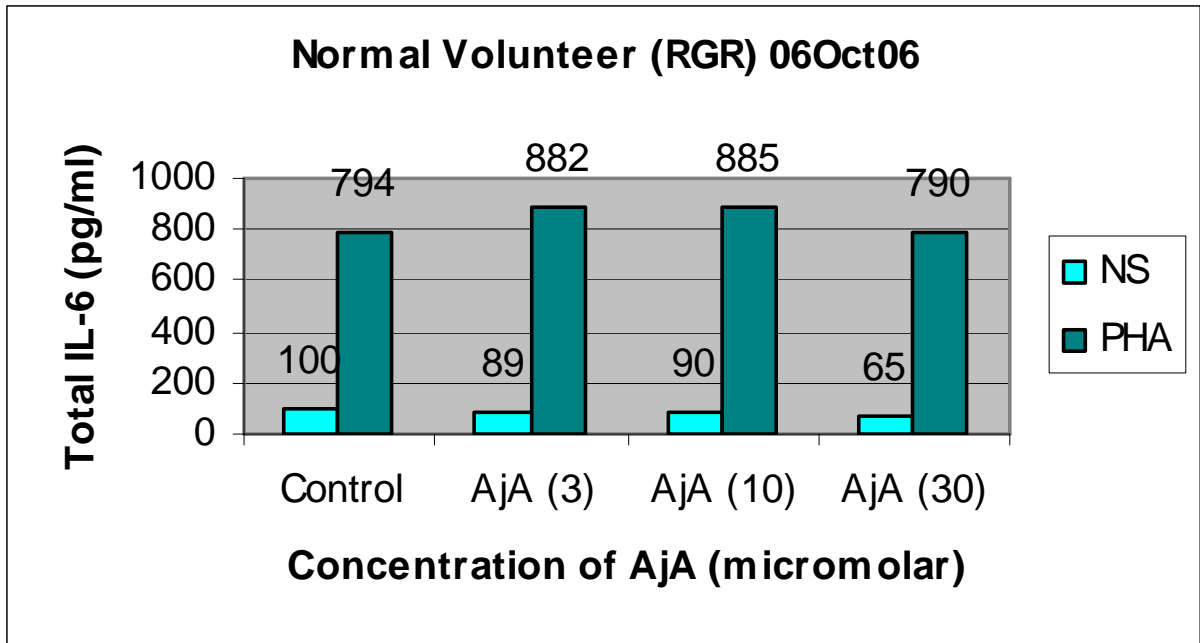


Figure B.7 IL-6 release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

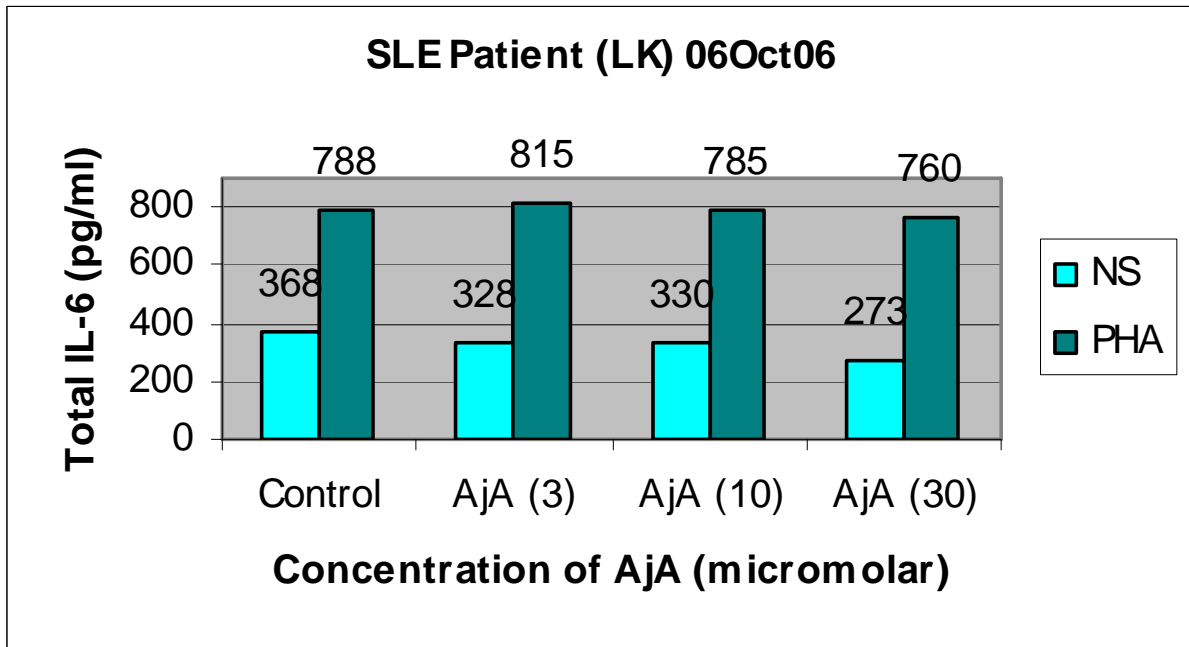


Figure B.8 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

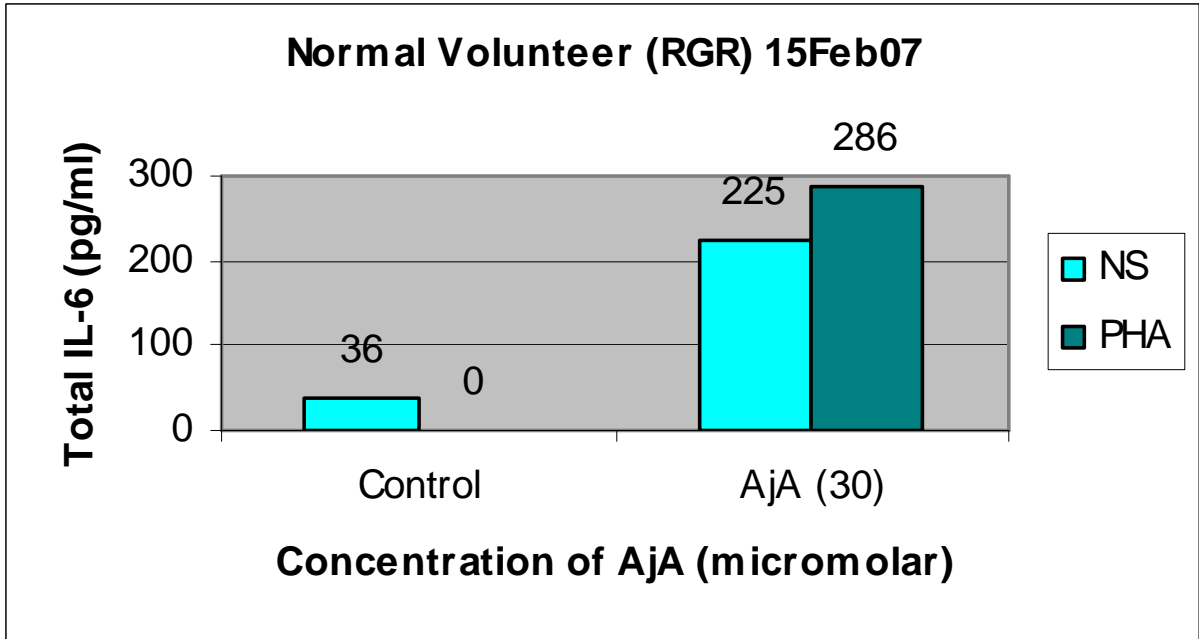


Figure B.9 IL-6 release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

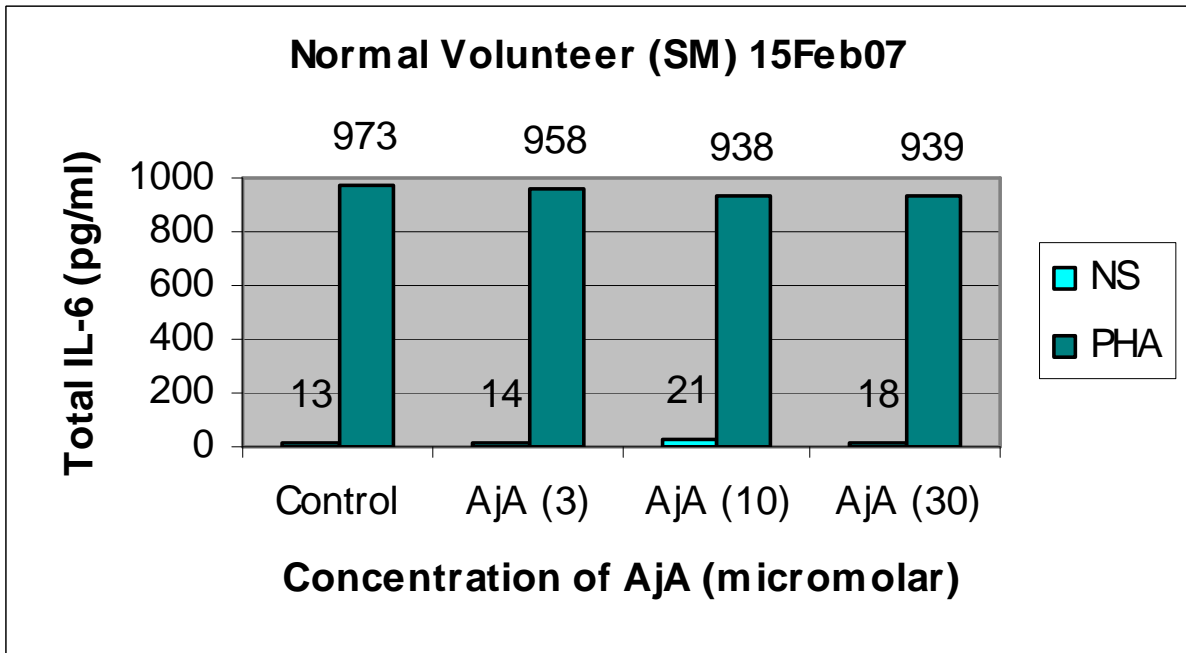


Figure B.10 IL-6 release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

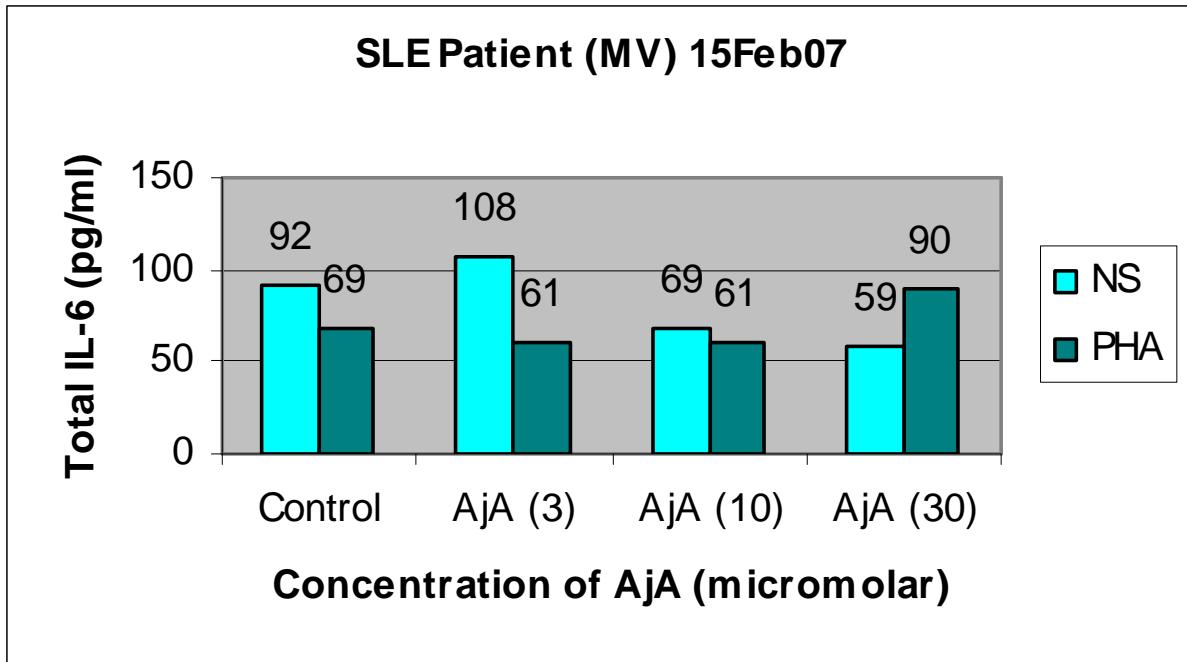


Figure B.11 IL-6 release from PBMC isolated from blood of an SLE patient. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IL-6 in supernatants measured by ELISA.

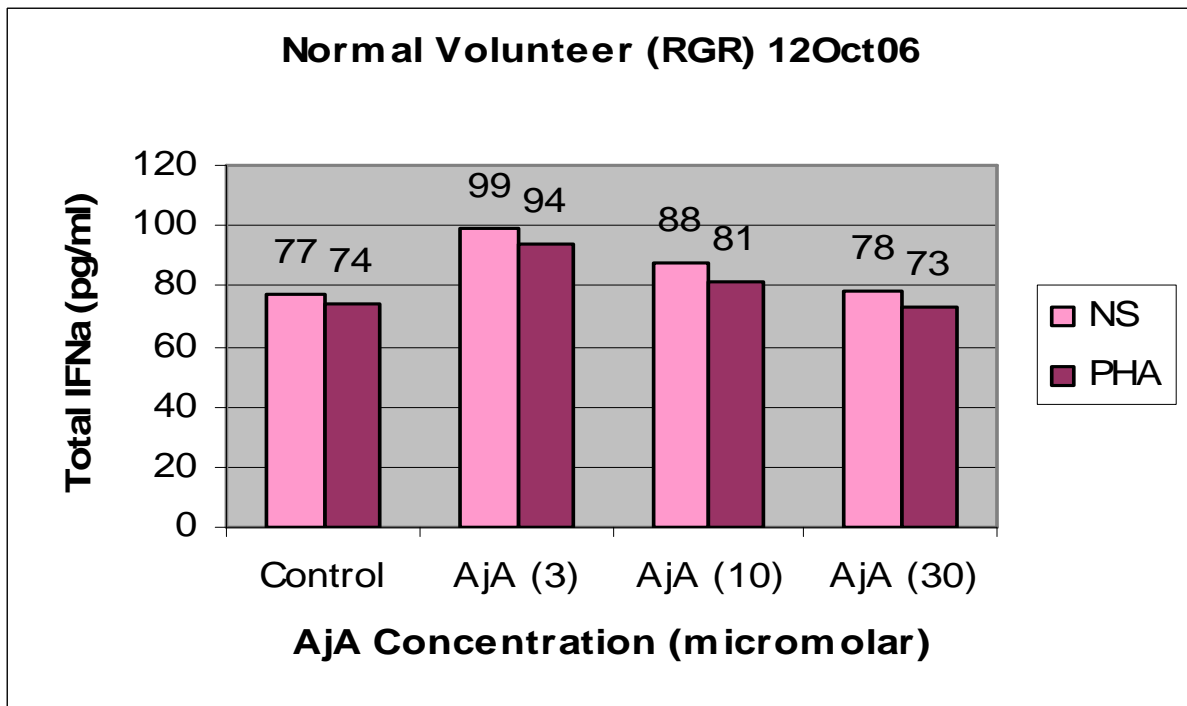


Figure C.1 IFN- α release from PBMC isolated from blood of a normal volunteer. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

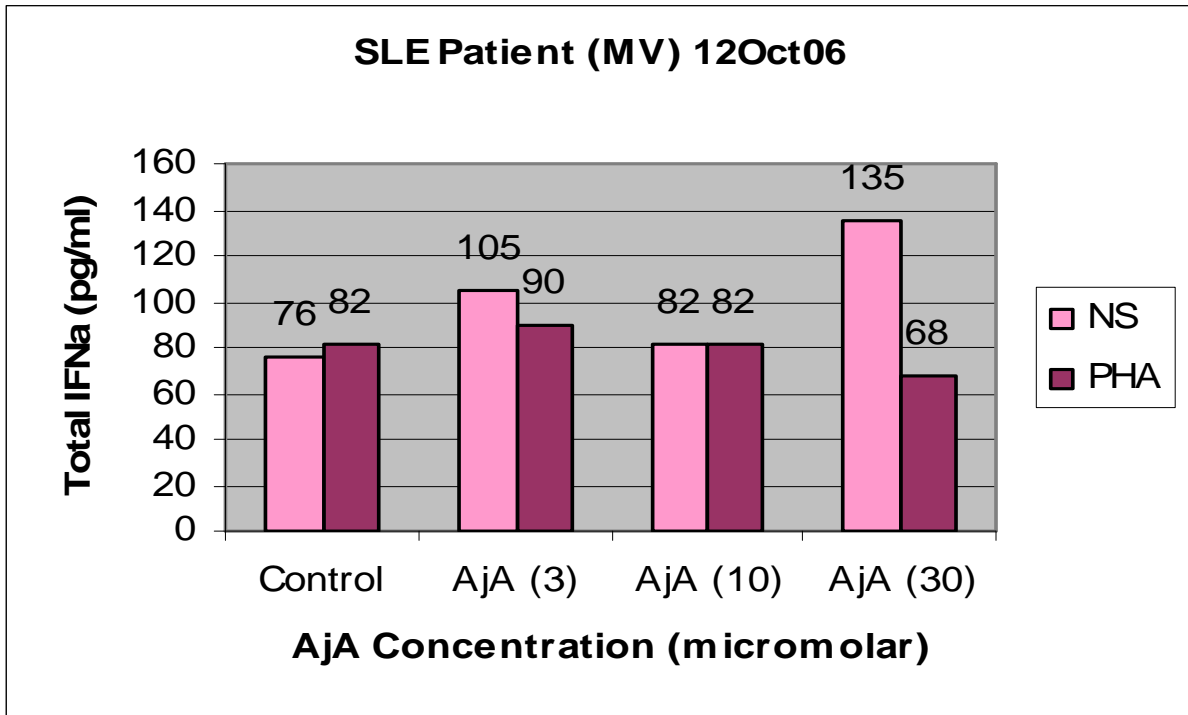


Figure C.2 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

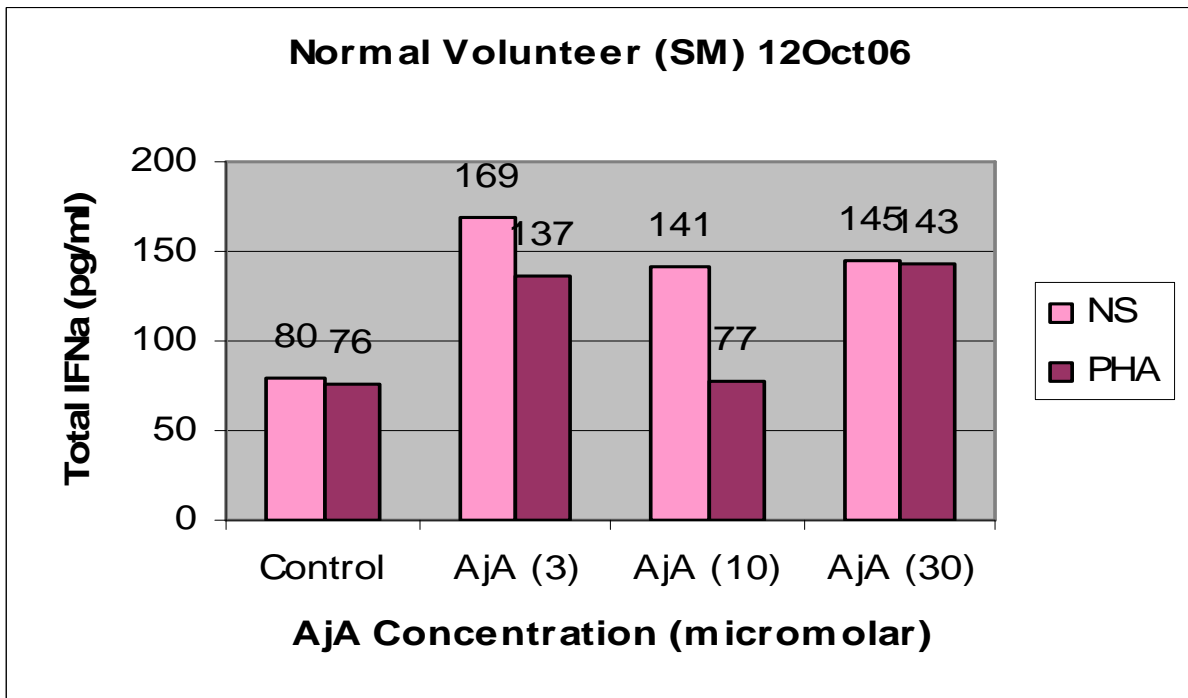


Figure C.3 IFN- α release from PBMC isolated from blood of a normal volunteer. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

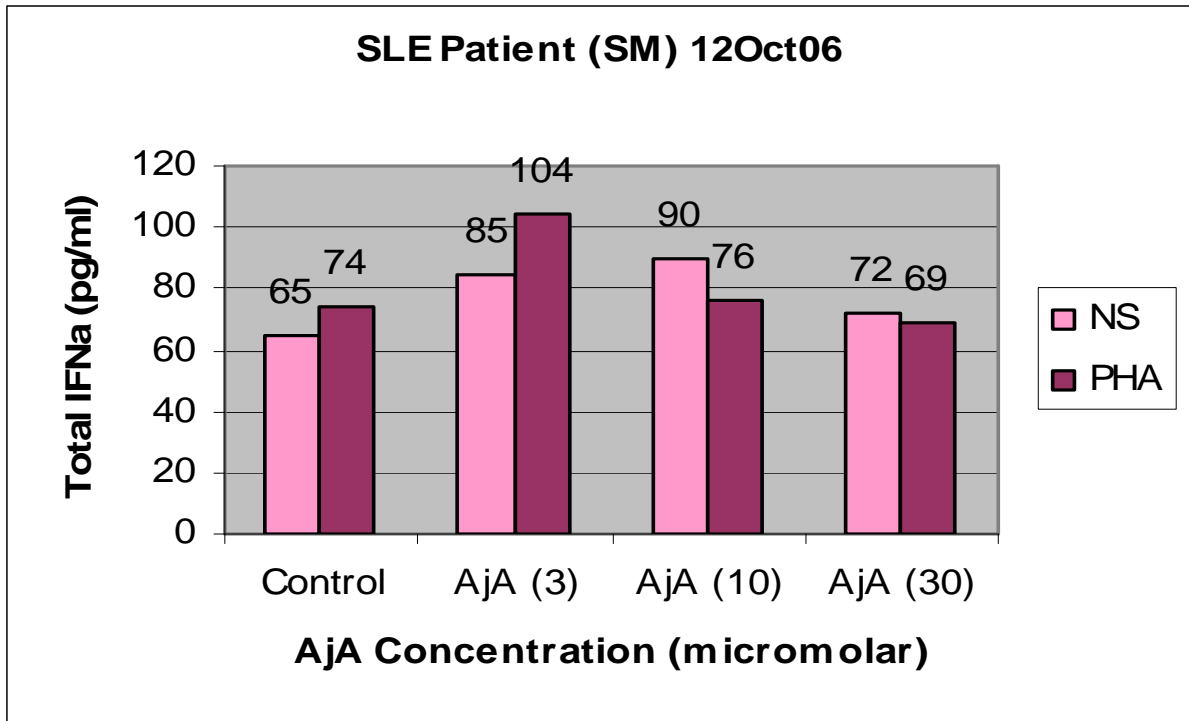


Figure C.4 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

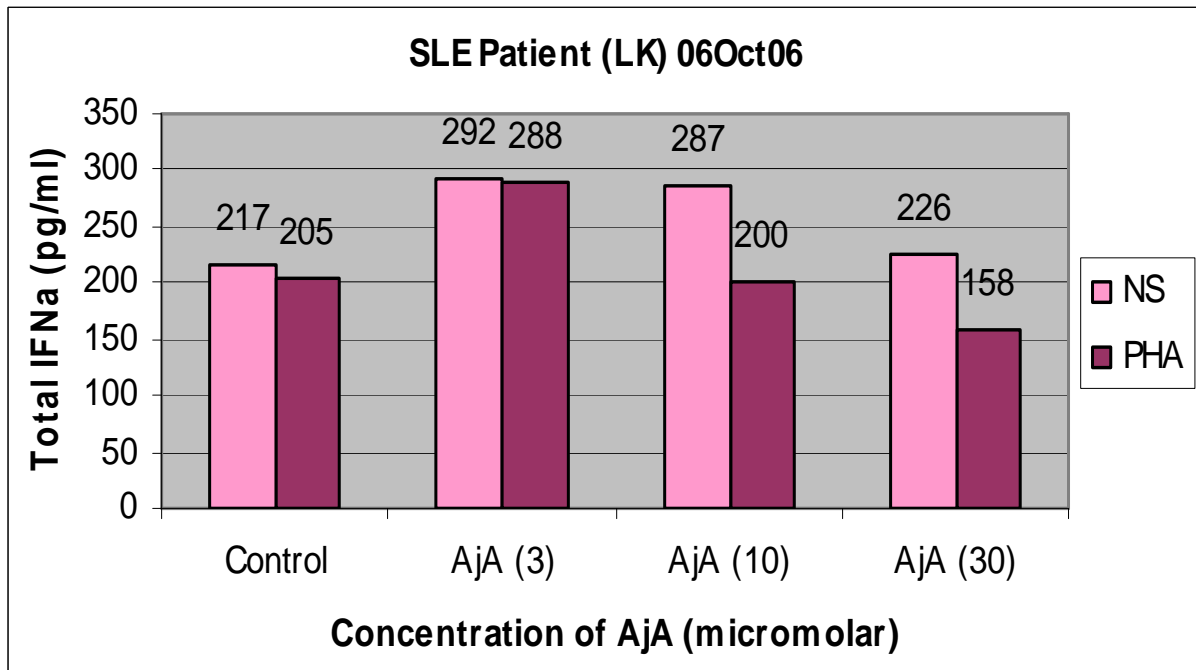


Figure C.5 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- α in supernatants measured by ELISA.

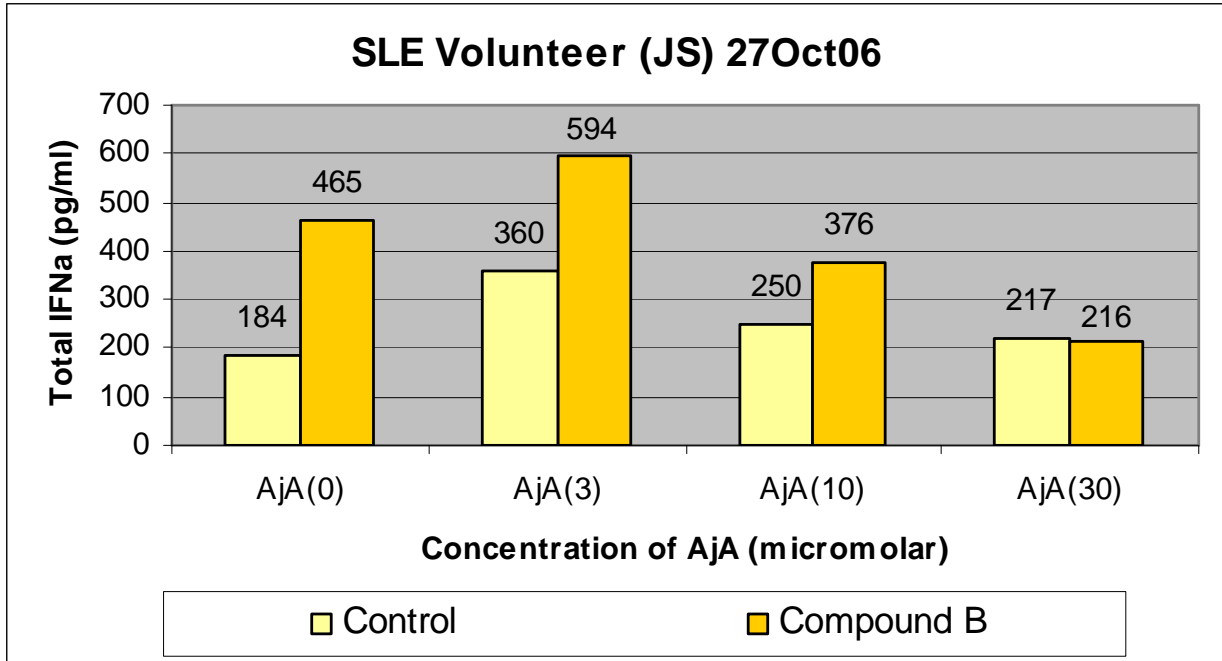


Figure D.1 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 4hr with Compound B (1/250). IFN- α in supernatants measured by ELISA.

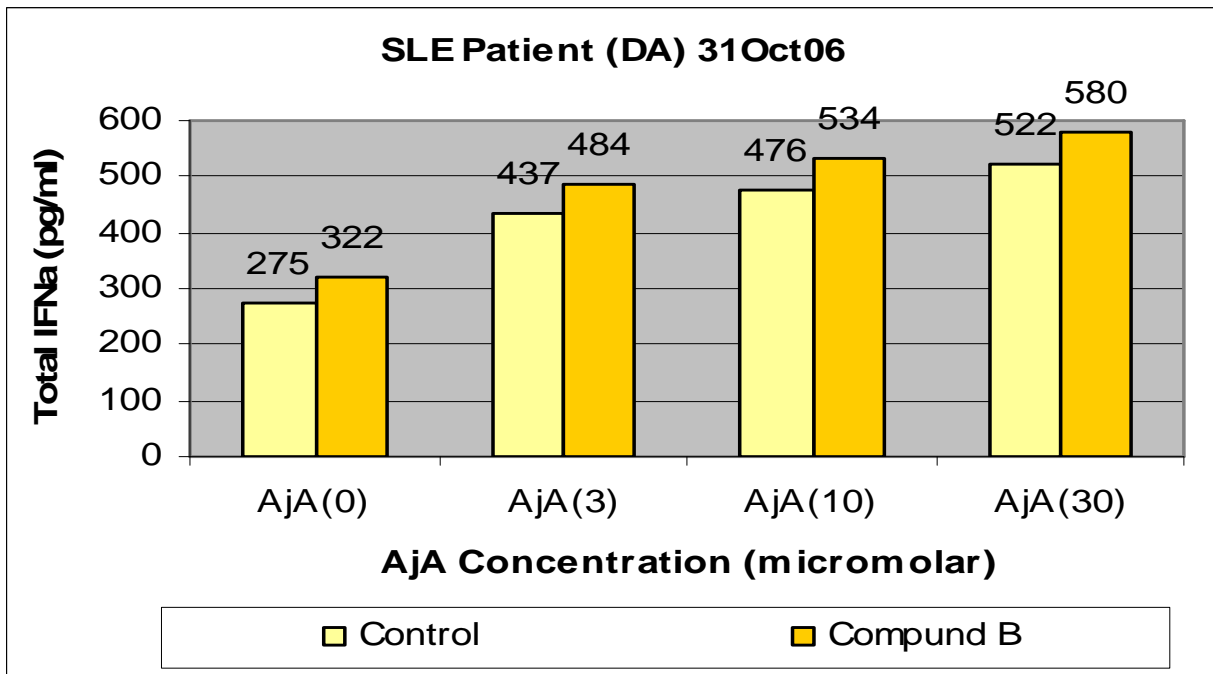


Figure D.2 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 4hr with Compound B (1/250). IFN- α in supernatants measured by ELISA.

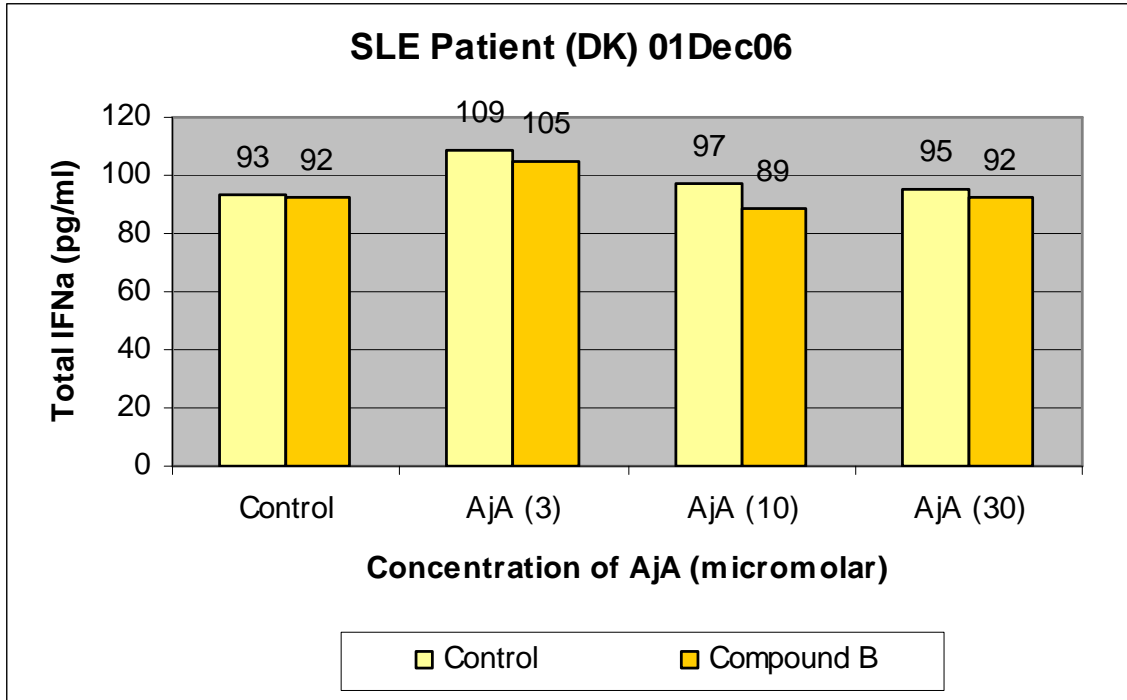


Figure D.3 IFN- α release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 4hr with Compound B (1/250). IFN- α in supernatants measured by ELISA.

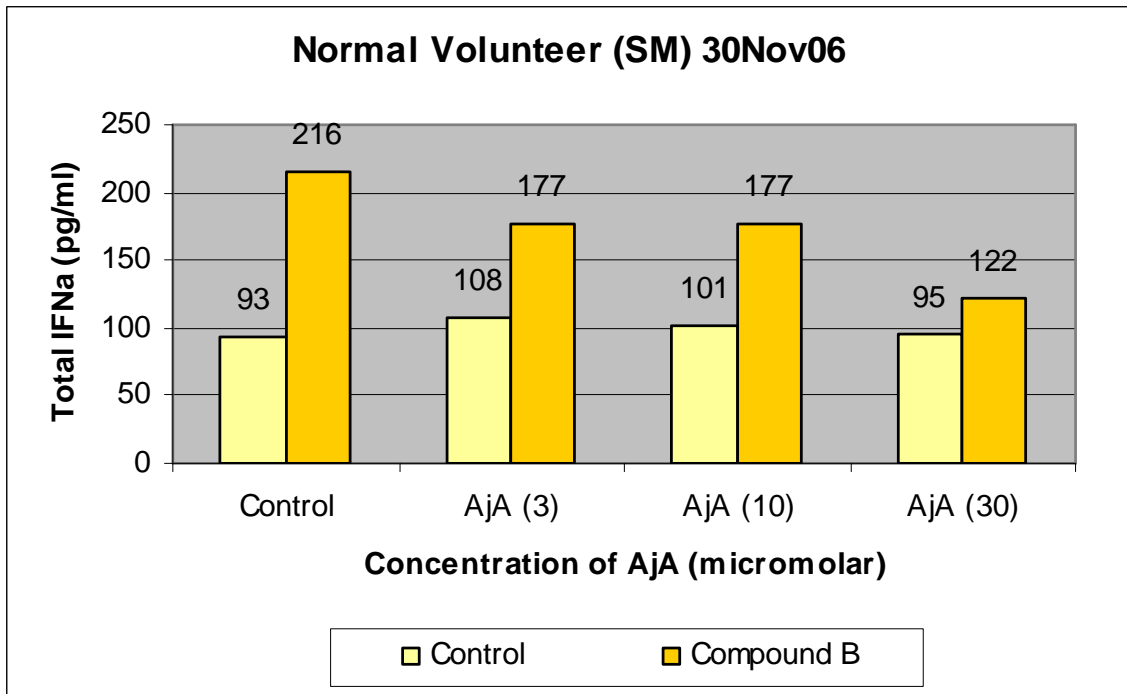


Figure D.4 IFN- α release from PBMC isolated from blood of normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 4hr with Compound B (1/250). IFN- α in supernatants measured by ELISA.

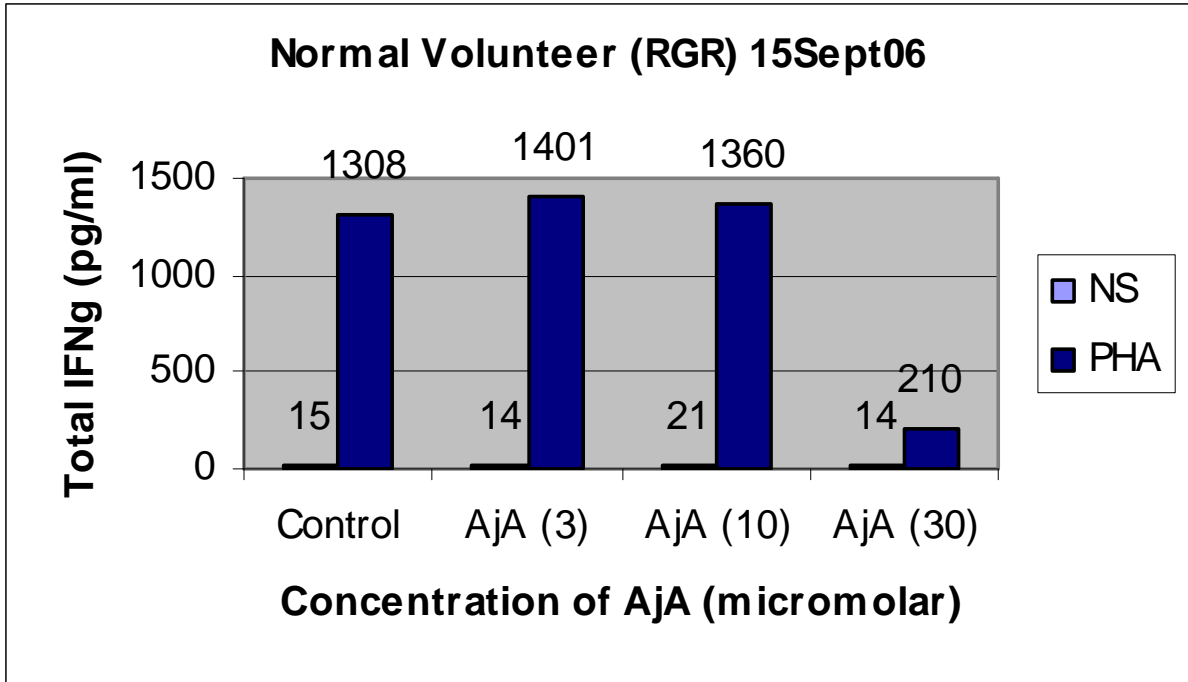


Figure E.1 IFN- γ release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

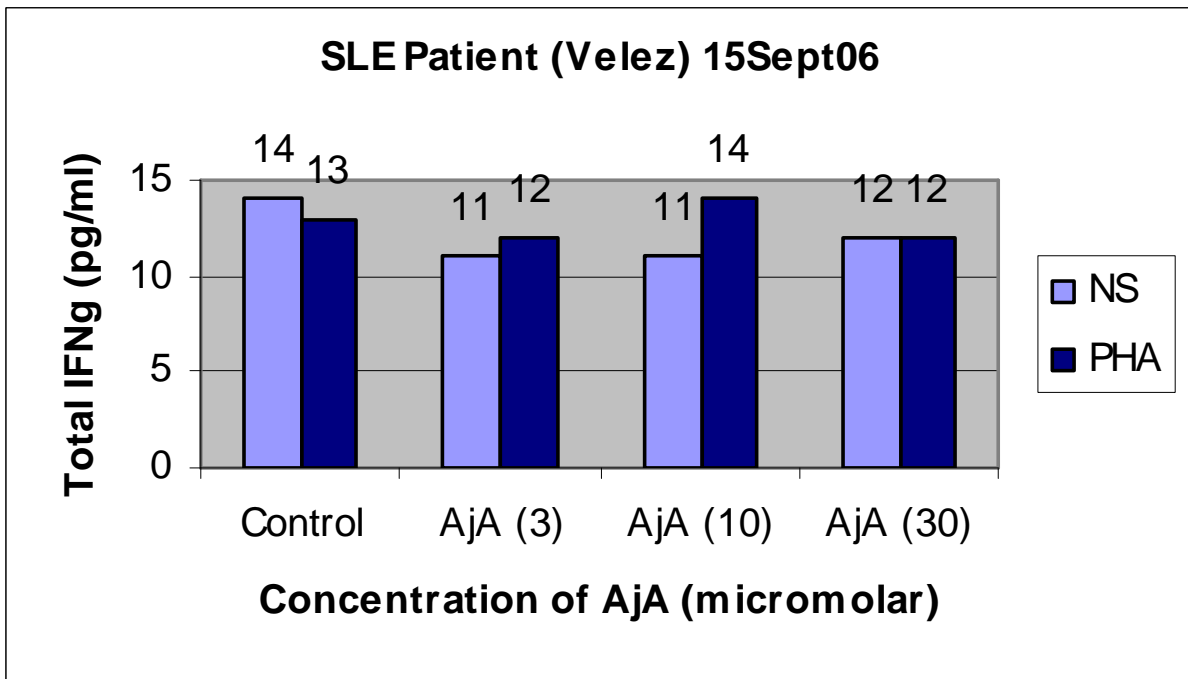


Figure E.2 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

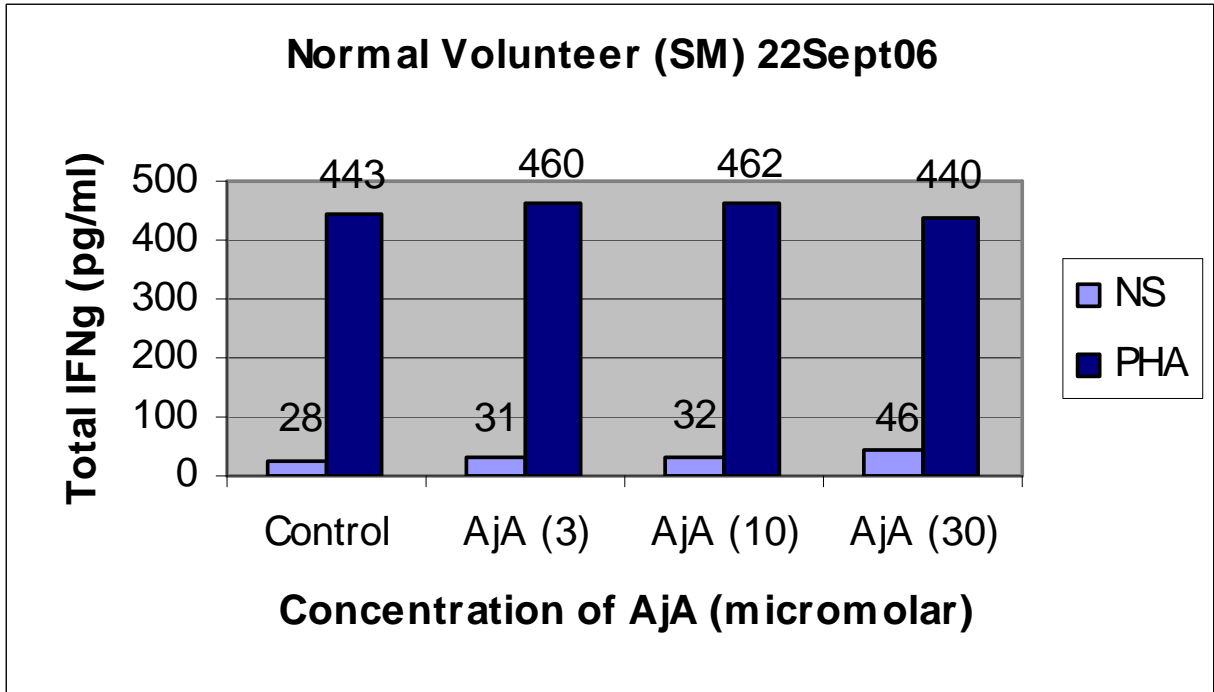


Figure E.3 IFN- γ release from PBMC isolated from blood of a normal volunteer. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

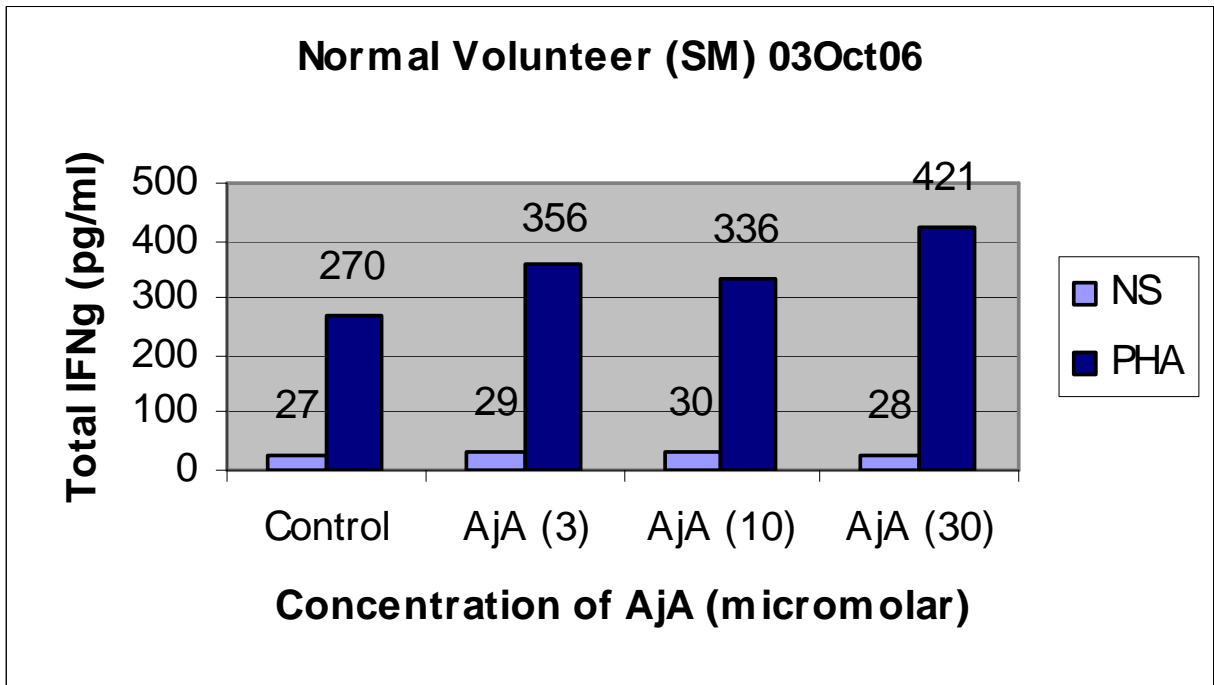


Figure E.4 IFN- γ release from PBMC isolated from blood of a normal volunteer. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

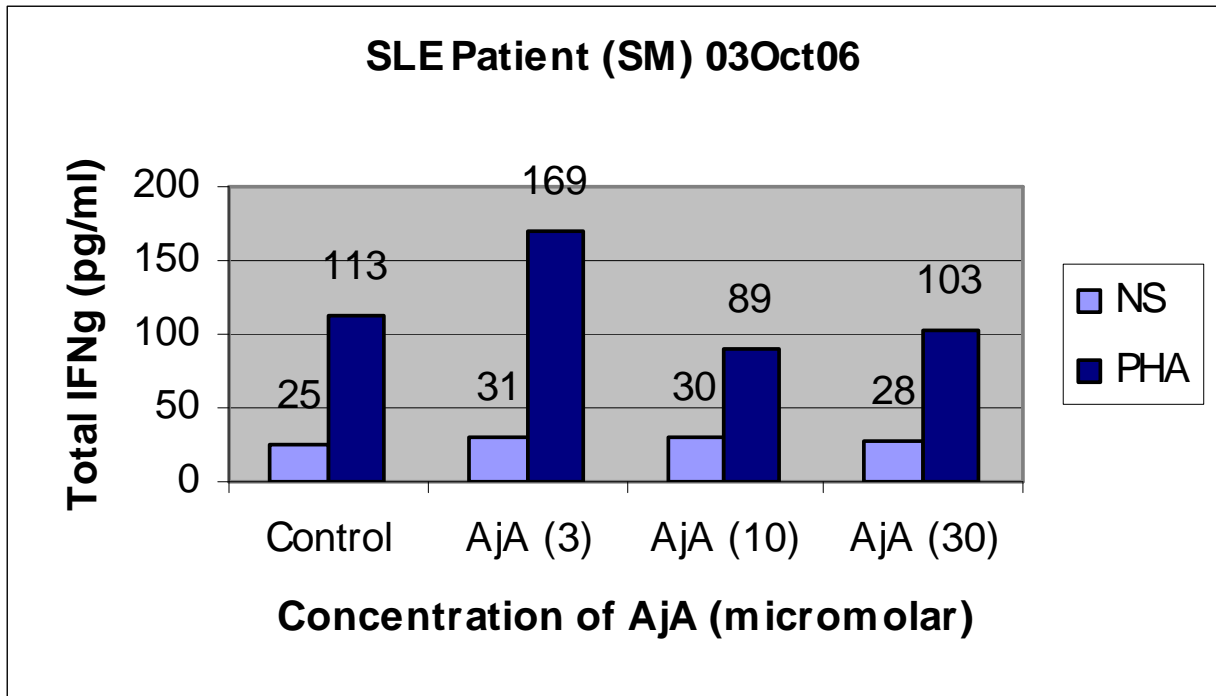


Figure E.5 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

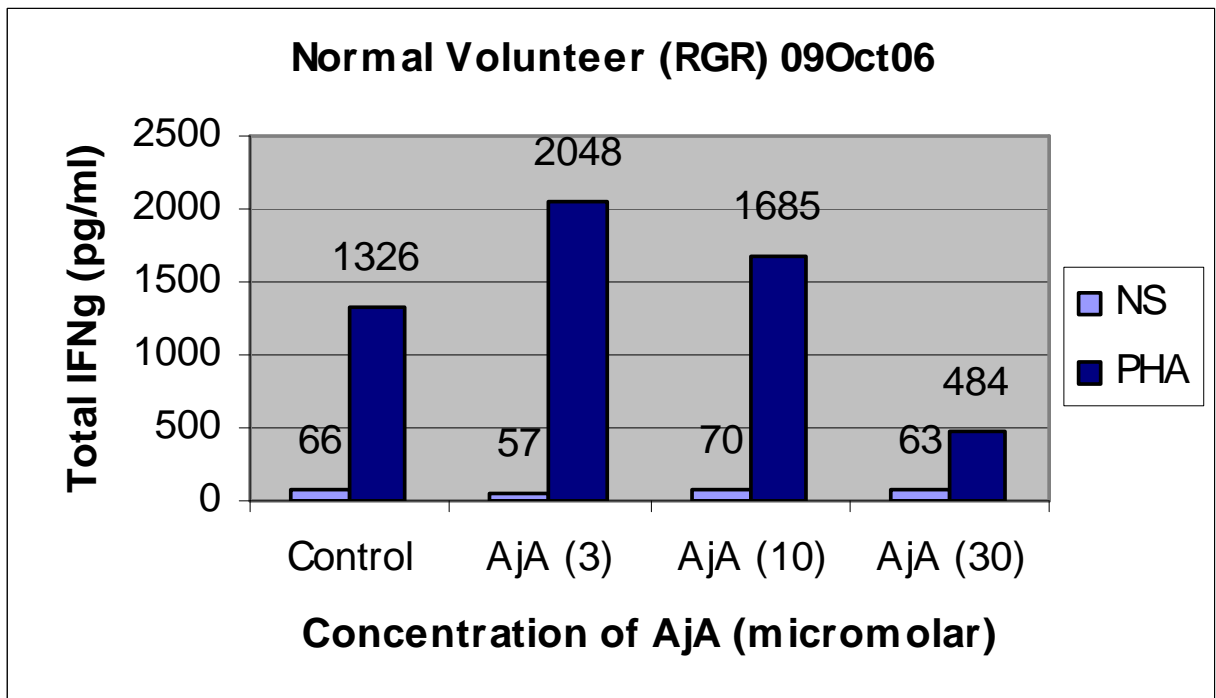


Figure E.6 IFN- γ release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

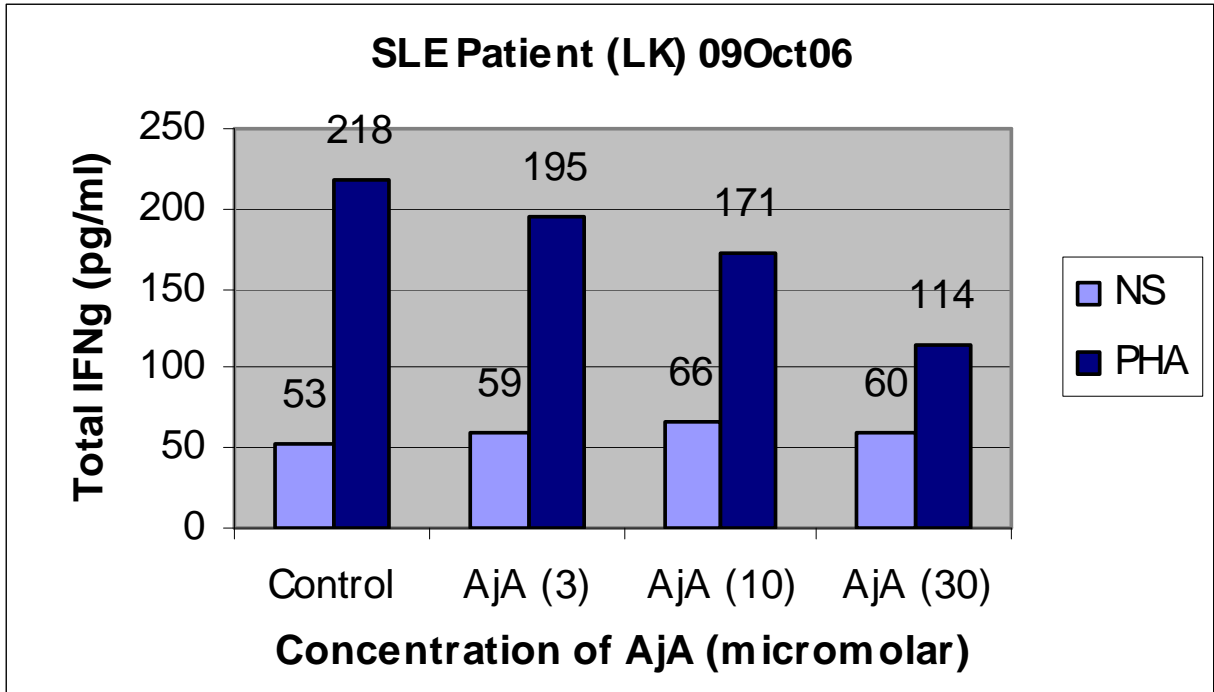


Figure E.7 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

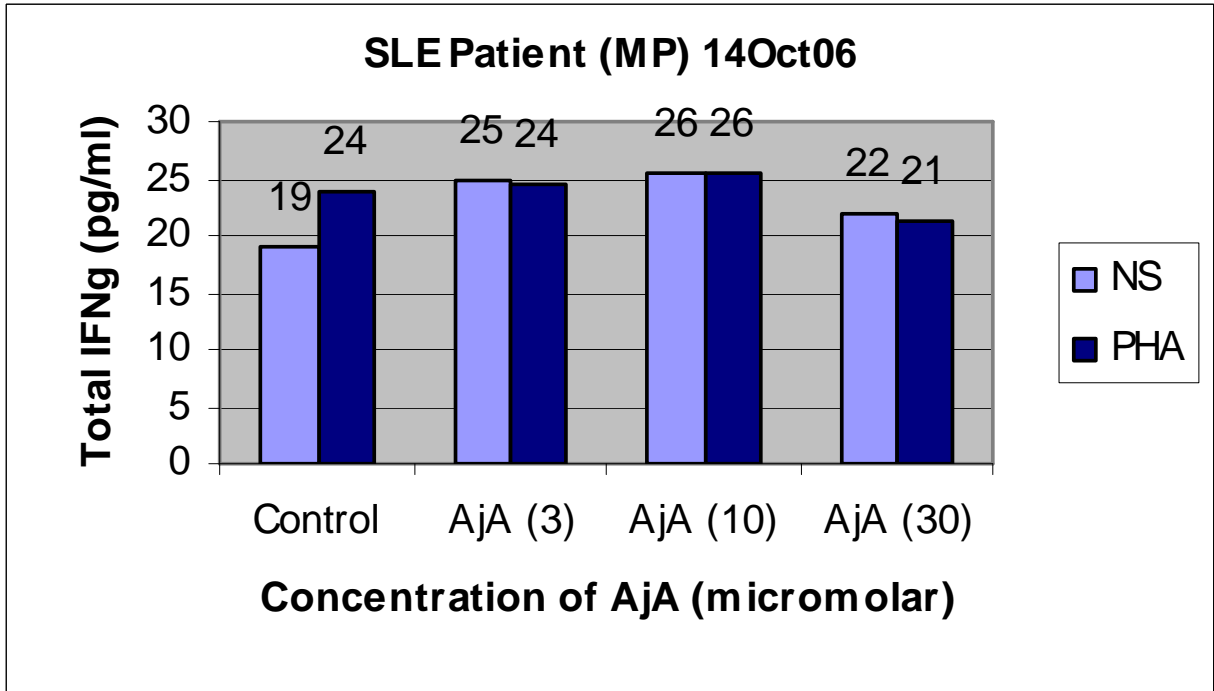


Figure E.8 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

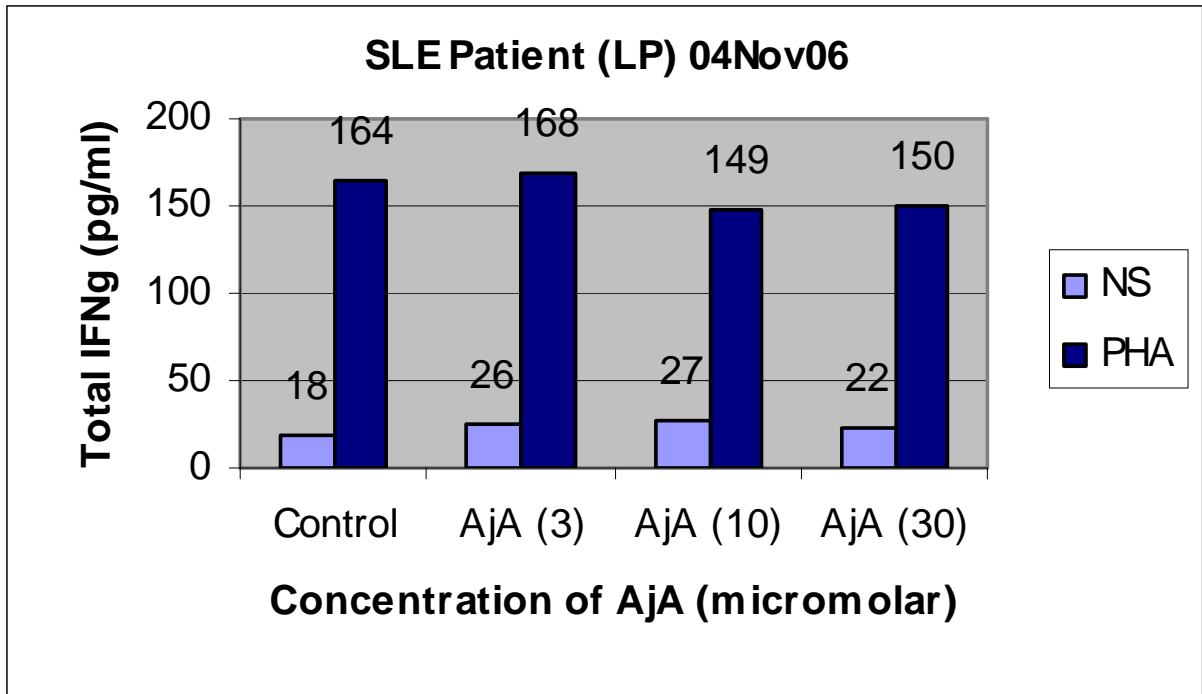


Figure E.9 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

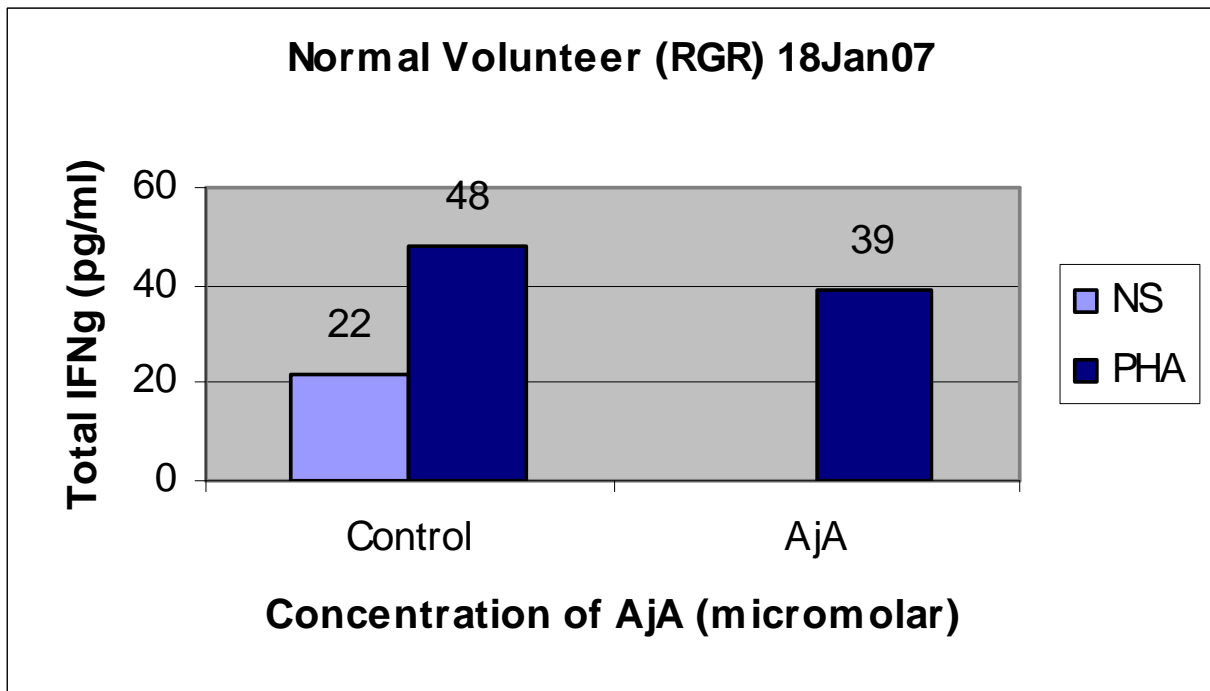


Figure E.10 IFN- γ release from PBMC isolated from blood of a normal volunteer. Cells exposed to AjA for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.

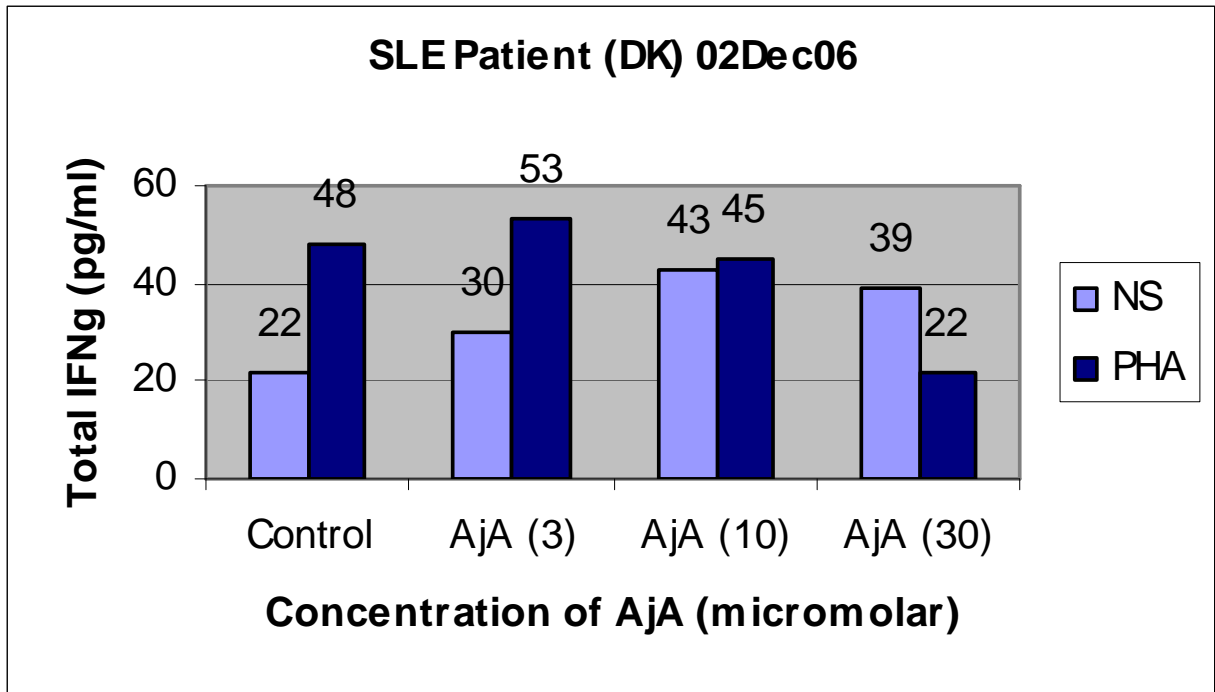


Figure E.11 IFN- γ release from PBMC isolated from blood of an SLE patient. Cells exposed to Aja for 60 min., then stimulated for 18hr with 10 μ M PHA. IFN- γ in supernatants measured by ELISA.