EFFECTS OF ADJUVANTS FOR HUMAN USE IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)-PRONE (NZB/NZW) F1 MICE.

Short title: adjuvant effect in SLE-prone mice

Elvira Favoino, Eleonora Isabella Favia, Liboria Digiglio, Vito Racanelli,

Yehuda Shoenfeld*†, Federico Perosa

Department of Internal Medicine (DIMO), Rheumatologic and Systemic Autoimmune Diseases, and Internal Medicine section, University of Bari Medical School, Bari, 70124, Italy.
*Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Aviv University, Tel-Hashomer 52621, Israel.
†Incumbent of the Laura Schwarz-Kipp Chair for Research of Autoimmune Diseases, Tel-Aviv University, Israel.

Address correspondence to: Federico Perosa, M.D., Ph.D., Department of Internal Medicine and Clinical Oncology (DIMO), University of Bari Medical School, Piazza G. Cesare 11, 70124 Bari, Italy.
Phone: +39-80-547-8891/+39-80-542-4600. Fax: +39-80-547-8820.
E-mail: federico.perosa@uniba.it

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Abreviations used: **Ab**: antibody/ies; **ALU**: aluminium hydroxide; BWF1: (New Zealand Black/ New Zealand White) F1; **BSA**: bovine serum albumin; **CFA**: complete Freund's adjuvant; **dsDNA:** double-stranded DNA; **ELISA**: enzyme-linked immunosorbent assay; **IFA**: incomplete Freund's adjuvant; **PBS-BSA**: phosphate buffered saline containing 0.5% BSA; **PBS-T20**: phosphate buffered saline containing 0.05% Tween 20; **SLE**: Systemic lupus erythematosus; **SQU**: squalene; **ssDNA:** single-stranded DNA; **UNT**: adjuvant untreated; **wk/s**: week/weeks.

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SUMMARY

The safety of 4 different adjuvants was assessed in lupus-prone BWF1 mice. Four groups of mice were intraperitoneally injected with incomplete Freund's adjuvant (IFA), or complete Freund's adjuvant (CFA), Squalene (SQU) or aluminum hydroxide (ALU). An additional group received plain PBS (UNT group). Mice were primed at week 9 and boosted every other week up to week 15. Proteinuria became detectable at week 17 (IFA group), 24 (CFA group), 28 (SQU and ALU groups) and 32 (UNT group). Different mean values were obtained among the groups from week 17 to 21 (week 17: one way Anova $p=0.016$; week 18 and 19: $p=0.048$; week 20 and 21: $p=0.013$) being higher in the IFA group than the others (Tukey HD post test $p<0.05$). No differences in anti-DNA Ab levels were observed among groups. Anti-RNP/Sm Ab developed at week 19 in only one CFA-treated mouse. Mean mouse weight at week 18 was lower in the ALU group than the IFA (Tukey HSD post test $p=0.04$), CFA ($p=0.01$) and SQU ($p<0.0001$) groups, while the mean weight in the SQU group was higher than in the IFA ($p=0.009$), CFA ($p=0.013$) and UNT ($p=0.005$) groups. The ALU group weight decreased by almost half between week 29 and 31, indicating some toxic effect of ALU in the late post-immunization period. Thus, SQU was the least toxic adjuvant as it did not i) accelerate proteinuria onset as compared to IFA; ii) induce toxicity as compared to ALU nor iii) elicit anti-RNP/Sm auto-Ab, as occurred in the CFA group.

INTRODUCTION

Systemic lupus erythematosus (SLE) is the prototype autoimmune disease, and can severely affect various different organs (i. e. the kidney, lung and nervous central system). The disease is still associated with high morbidity and mortality rates, despite the use of a wide range of drugs that can control symptoms, delay progression and/or improve the quality of life, but never bring about a complete cure. Among the most innovative therapeutic approaches, inducing B cell depletion by the passive administration of anti-CD20 monoclonal antibodies (mAb) seems to show promise in counteracting SLE activity, as demonstrated in mice [1] and humans (albeit in open non-controlled clinical trials) [2-7].

We previously characterized 11 7-mer Rituximab-specific peptides [8, 9], some of which can induce an anti-CD20 response in mice, with biological effects similar to those obtained with the passive administration of anti-CD20 mAb [8, 9]. The properties of these peptides led us to consider them as a potential vaccine in SLE. The mouse strain (New Zealand Black x New Zealand white)F1 (BWF1) can be considered a suitable model for testing the efficacy of the CD20 mimotope peptide, since it spontaneously develops a human-like SLE characterized by the presence of an autoimmune glomerulonephritis (indicated by the appearance of proteinuria) and the presence of lupus-specific auto-Ab [10]. However, to obtain an efficient response, a peptide (which behaves as a hapten) has to be coupled to a macromolecule (carrier) to achieve an adequate presentation [11], and incorporated with an appropriate adjuvant.

Adjuvants have greatly enhanced the efficacy of active immunotherapy thanks to their ability to stimulate inflammatory and adaptive immune responses in a non-specific fashion [12-14]. Aluminum hydroxide (ALU) and mineral (or organic) oils are the adjuvants most commonly employed in animals and humans. ALU is currently used in human vaccines

against diphtheria, tetanus, pertussis, Haemophilus influentiae B, Hepatitis A/B, human papilloma virus, and Anthrax [15-17]. Oil adjuvants include pristane (2,6,10,14 tetramethylpentadecane), incomplete Freund's adjuvant (IFA) (an emulsion of mineral oil Bayol F and water), complete Freund's adjuvant (CFA) (composed of inactivated and dried mycobacteria added to Bayol F), and MF59, consisting of an emulsion of the organic oil squalene (SQU) and water (~2 % w/v). With the exception of pristane and CFA, only used in animals, IFA has been employed in humans against influenza [18], is currently being tested in experimental HIV [19] and as a tumor vaccine [20], while MF59 is included in seasonal influenza and influenza A (H1N1) vaccine preparations [[21, 22]]. However, even the last generation of adjuvants (SQU adjuvants), like the early one [23], specifically elicited anti-nuclear Ab in non-autoimmune prone Balb/c mice [24] and pathogenic anti-phospholipid antibodies in C57/B6 mice [25]. Indeed, Balb/c mice receiving adjuvants developed either an autoimmune glomerulonephritis [26] or auto-Ab to RNP/Sm and to single-stranded DNA (ssDNA) [23], the specificity profile/pattern of these auto-Ab being dependent on the type of adjuvant used [27].

Recently, a clinical syndrome referred to as "autoimmune/autoinflammatory syndrome induced by adjuvant" (ASIA), encompassing 4 different clinical entities, namely siliconosis [28, 29], the "gulf war syndrome" [30], macrophagic myofasciitis syndrome [31] and post-vaccination phenomena [29, 32-34] has been described in humans. The symptoms are overlapping and have been attributed to the likely effect of adjuvants [reviewed in [35-38]]. All the above findings raised concerns as to whether adjuvants alone could be harmful in our animal model of SLE, by acting as a sort of autoimmune disease "activator", and so reversing the potential beneficial effect of a CD20-mimotope peptide vaccine.

To identify the least harmful adjuvant, since no definite data are yet available on the effects of adjuvants on human-like SLE prone mice BWF1, we evaluated the effects of IFA,

CFA, ALU and SQU on the disease course in this mouse strain.

MATERIALS AND METHODS

Reagents and antibodies

Keyhole Limpet Hemocyanin (KLH) was purchased from Calbiochem (San Diego, CA, USA). IFA and CFA were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). SQU adjuvant (AddaVax) and ALU adjuvant (Alhydrogel) were purchased from Invivogen (San Diego, CA, USA). Double-stranded calf thymus DNA was purchased from Sigma (St. Louis, MO, USA). RNP/Sm antigen was purchased from Arotec Diagnostics Limited (Wellington, New Zealand). Phycoerythrin-cyanin 5.1 (PE-Cy5)-conjugated anti-mouse CD3 Ab, PE-conjugated anti-mouse CD8 Ab, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and CD19 Ab were purchased from BD Pharmingen (Franklin Lakes, NJ, USA).

Mice and immunization strategy

Nine-week-old BWF1 female mice were purchased from Harlan Laboratories (San Pietro al Natisone, Italy). Mice were kept at the University of Bari with a 12 h light/dark cycle and experiments were performed in accordance with institutional and internationally approved guidelines on animal research. Animals study protocols were approved by the University of Bari Medical School ethical review Committee.

BWF1 mice were treated with adjuvants that are commercially available for human use (Table 1), namely IFA, SQU, ALU, which were mixed with KLH before injection. Immunogen (200 µl of adjuvant emulsion/injection) was prepared by mixing 100µl of PBS containing 100µg KLH with an equal volume of adjuvant. Four groups of mice (4 mice per group) were primed at the 9th week (wk) (when no proteinuria was detected in any of them)

with IFA, CFA, SQU, or ALU adjuvant. Boosters were given fortnightly (up to the 15th wk) until proteinuria ≥100 mg/dl was detected in at least 1 mouse in each group. Two additional groups of mice treated with PBS only (adjuvant untreated group; UNT), and with CFA, served as the negative and positive [39] control groups. Groups with only one mouse left were excluded from further evaluations.

Blood was collected every two wks by an orbital technique from the ophthalmic venous plexus. An aliquot of blood was rapidly mixed with 900 µl of PBS containing 1.8 mg K3-EDTA and another aliquot was used for serum recovery. Sera were stored at -80°C until used, while EDTA blood was immediately used for white blood cell (WBC) counts and cytofluorimetric assays. Mice were monitored for proteinuria, serum levels of anti-ssDNA and anti-RNP/Sm Ab, and weight.

Proteinuria measurement

Urine samples were collected weekly and proteinuria was assessed with the Urine Strip Test (Dyaset s.r.l, Portomaggiore, Italy). Urine was colorimetrically graded on a scale from 0 to 4+, corresponding to the following protein concentrations: 0, negative or trace (15-20 mg/dl), 1+ (30 mg/dl), 2+ (100 mg/dl), 3+ (300 mg/dl), or 4+ (>2000 mg/dl). Mice were considered to show proteinuria if two consecutive urine samples were scored ≥1 (30 mg/dl).

Analysis of serum anti-DNA Ab and anti-RNP/Sm Ab.

Anti-ssDNA Ab were detected by ELISA as previously described [40], with minor modifications. Briefly, polyvinylchloride 96-well plates were precoated with 50 µl of poly-l-lysine (Sigma) by a 1 hr incubation at 37°C. Then, wells were washed three times with PBS and incubated with ssDNA (5 µg/ml), previously denaturated by boiling calf thymus DNA for 10 min and cooling on ice for 15 min. Following a 12 h at 4°C, wells were washed once with PBS containing 0.05% Tween 20 (PBS-T20) and blocked with PBS containing 0.5% BSA

(PBS-BSA). Serum samples (diluted 200 times in PBS-BSA) were added to the wells and incubated for 4 h at 25°C. Wells were washed three times with PBS-T20. Bound IgG were detected by sequential incubation with HRP-conjugated xeno-Ab to the Fc portion of mouse IgG (1 h incubation at 25°C) and o-phenylenediamine (0.5 mg/ml; 100 µl/well); color development was stopped by adding 100 µl 2 N H_2SO_4 and the absorbance at 490 nm was read with the Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Background binding was determined from the absorbance generated in wells with blocking solution alone. Specific binding was determined by subtracting the background absorbance from the absorbance in the experimental wells. Results were expressed as units/ml extrapolated from a calibration curve constructed using different dilutions of a positive control serum.

Anti-double-stranded DNA (dsDNA) Ab were detected by ELISA as previously described [41, 42], with minor modifications. Briefly, calf-thymus DNA was passed through a 0.45 µm nitrocellulose Millex syringe filter (Millipore Corp., Bedford, MA, USA) to remove any ssDNA fragments. Then, 50 µl of dsDNA solution (50µg/ml per well) was added to plates pre-coated with poly-l-lysine, and incubated overnight at 37°C. Afterwards, plates were washed and the assay was continued as described above for anti-ssDNA Ab quantification.

Anti-RNP/Sm Ab were detected in ELISA, using microtiter plates coated with RNP/Sm antigen after overnight incubation at 4°C of 50 µl/well of PBS solution containing 5 µg/ml of antigen. Then wells were washed once with PBS-T20 and blocked with PBS-BSA. Serum samples (diluted 200 times in PBS-BSA) were added to the wells and incubated for 3 h at 25°C. Wells were washed three times with PBS-T20. Detection of bound IgG and color reactions was performed as described above for the anti-DNA Ab assay.

Flow cytometry analysis

The WBC count per µl of EDTA/whole blood was determined with the Sysmex KX-

21N™ Automated Hematology Analyzer (Lincolnshire, IL, USA)**.** Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. One hundred µl of EDTA-blood were layered on 300 µl of Ficoll in an eppendorf tube. Following a 30 min centrifugation (500 *x* g) at 20°C, the lymphocyte layer was recovered and washed twice in PBS. Cells ($2x10^4$) were incubated for 30 min at 4°C with anti-mouse CD3 (PE-Cy5), CD8 (PE), CD4 (FITC), and CD19 (FITC) Ab, blocked with rabbit IgG (50 µg/ml) and washed with PBS-BSA, fixed in 2% formaldehyde and analyzed by flow cytometry (Cyan, Beckman Coulter, Brea, CA, USA). Lymphocyte subset counts were calculated by multiplying the total lymphocyte count by the percentage of **e**ach cell type. Results are expressed as the mean number of cells ($1x10^3$/µl).

Statistical analysis

All statistical analyses were performed using SPSS software for windows v.20. Differences between groups in terms of the arithmetic mean of proteinuria, mouse weight, and anti-DNA titer were analyzed by one-way ANOVA. In cases of statistically significant differences, set at $p<0.05$, the Tukey HSD post test was performed for comparisons.

RESULTS

BWF1 mice spontaneously develop an autoimmune disease resembling human SLE. In these mice, anti-DNA Ab appear in their sera between the 16^{th} and 24^{th} wk, and proteinuria between the 28^{th} and 32^{nd} wk, affecting 80% of mice at 35^{th} wk [43].

Two accidental (not disease related) deaths were recorded: one in the IFA group at wk 17 and 1 in the ALU group at wk 19.

Mice were primed at the 9^{th} wk and immunization was discontinued from the 17^{th} wk, when a proteinuria score ≥2 was detected in one mouse (IFA group) (Fig. 1). In the subsequent wk, proteinuria appeared significantly earlier in the IFA group than in the others

(Fig. 1). Indeed, in the IFA group, a proteinuria mean score of 1 was observed as early as at wk 17 and increased to 2 and then 2.5 at wks 27 and 28. In the CFA group, proteinuria became detectable at wk 24 (mean score 0.5), and reached 1, 1.5 at wks 27 and 28. Proteinuria showed a similar trend in the SQU and ALU groups, becoming detectable at wk 28 (SQU: 0.5; ALU: 1) and increasing from wk 29 (SQU: 1.7; ALU: 2.3), to wk 31 (SQU: 2; ALU: 2.5) (representative results are shown in Fig. 1). In the UNT group, proteinuria became detectable at wk 32 (mean score: 0.5), then increased to 1.5 and 3 by wks 34 and 35. Overall, the mean proteinuria value was statistically different among the groups from wk 17 to 21 (wk 17: one-way Anova $p=0.016$; wk 18 and 19: $p=0.05$; wk 20 and 21: $p=0.013$). The Tukey HSD post-test indicated that proteinuria was statistically higher in the IFA group than in the other groups at wk 17 ($p=0.026$), 18 and 19 ($p=0.05$), and at wk 20 and 21 ($p=0.021$), while no differences were observed among the other groups ($p>0.05$). After the 21st wk, no significant differences in proteinuria were observed among groups up to wk 35. Overall, the results indicate that IFA not only accelerated the onset but also the progression of proteinuria in the 5 wks following the last injection of adjuvant, as compared to all the other groups, while CFA, SQU and ALU affected the disease course to a lesser extent (or not at all) as compared to the UNT group.

To define whether the type of adjuvant had any influence on the development of anti-ssDNA, anti-dsDNA and anti–RNP/Sm Ab, these Ab were evaluated from wk 9 up to wk 32. Fig. 2 shows that no significant differences were detectable in the titers of anti-ssDNA (Fig. 2A) or anti-dsDNA Ab (Fig. 2B) in the adjuvant treated groups as compared to the UNT mice (One-way Anova $p>0.05$), while anti-RNP/Sm Ab were not detected in any of the mice in the SQU and ALU groups (Fig. 2C). One mouse only, in the CFA group, developed these Ab early, at the 19th wk and the titer reached a plateau at the 29th wk. Anti-RNP/Sm Ab levels became barely detectable in one mouse in the UNT group at wk 32.

Based on previous studies indicating that adjuvant immunization may have an influence

on mouse weight changes [44], this parameter was monitored before immunization and weekly, after wk 18, when a visible difference in mice weight became evident. The increased weight observed up to wk 18 was very likely attributable to physiological growth, as similar changes were observed in the UNT mice. However, a statistically significant difference was recorded among groups at wks 18 (one way Anova $p<0.001$), 19 ($p=0.01$), 20 ($p=0.02$), 22 ($p=0.01$), and at wks 29 ($p=0.037$), 30 ($p=0.008$) and 31 ($p=0.035$). Specifically, at the 18th wk, the mean weight in the ALU group was lower than in the IFA (Tukey HSD post test $p=0.04$), CFA ($p=0.01$) and SQU ($p<0.0001$) groups, while the mean weight in the SQU group was higher than in the IFA ($p=0.009$), CFA ($p=0.013$) and UNT ($p=0.005$) groups. No significant differences in the mean weight were found between mice treated with CFA and IFA ($p=0.98$) and the control group ($p=0.68$), or between the latter and ALU treated mice ($p=0.28$). The ALU group mouse weight remained statistically lower than that of the CFA and SQU groups up to wk 22. In subsequent weeks up to wk 28, no statistically significant differences were observed in mouse weight among the groups. After the 28th wk, the mean weight of the ALU-treated mice decreased from 39 g to 28 g (at wk 31) (Fig. 2). This effect was likely specifically due to ALU, as no similar marked changes were observed in UNT mice or mice treated with an adjuvant other than ALU.

Cytofluorimetric analysis of peripheral blood lymphocytes from adjuvant treated mice (Fig. 4, closed symbols) showed no significant changes/differences in the number of CD4, CD8 and CD19 positive cells as compared to UNT mice (Fig. 4, open symbols) for all the monitoring period, indicating no specific effect of adjuvant in the changing lymphocyte sub population distribution.

DISCUSSION

This explorative study evaluated the effect of different adjuvants for human use in

BWF1, and showed that IFA, more than CFA, SQU and ALU, markedly accelerated the onset of proteinuria (and hence of glomerulonephritis). It is of interest that the differences in proteinuria between the groups were not associated to the anti-ssDNA and anti-dsDNA Ab titers, which were not statistically different. These findings are in agreement with previous observations by Gilkeson *et al.* [45], who showed that immunization of BWF1 mice with *Escherichia coli* DNA (in association to methylated BSA and adjuvant) attenuated murine SLE, although these mice developed pathogenic anti-DNA Ab earlier, and at higher titers, than control mice (immunized with BSA and adjuvant).

As to anti-RNP/Sm Ab levels, a previous study was unable to demonstrate the presence of these lupus-specific Ab in BWF sera [46]. Even so, the lack of detection of anti-RNP/Sm Ab in almost all adjuvant-treated mice was still an unexpected finding, considering i) the documented ability of CFA and mineral oil adjuvants to elicit anti-RNP/Sm Ab in non-autoimmune Balb/c mice [24] and ii) the previous detection of Sm-derived peptide-specific $CD4^+$ T cells in BWF mice [47]. The lack of an anti RNP/Sm response indicates that none of the adjuvants (but CFA in one mice) were able to overcome the genetic resistance to the development of these lupus-specific auto-Ab even in this lupus-prone mouse model. Overall, these data suggest that the accelerated proteinuria observed in the IFA mice was unrelated to either auto-Ab to DNA or to RNP/Sm.

A recent study by Bassi *et al.* [39] reported a similar fairly time lapse between the appearance of proteinuria in CFA-treated mice and in the UNT mice group (about 8 wks) to the time observed in our study. Even so, they found that BWF1 mice treated with CFA developed anti-dsDNA Ab together with proteinuria earlier, and at higher titers, than BWF1 mice treated with PBS [39]. One possible explanation for these different results may be the different immunization routes employed, as we used an intraperitoneal route while Bassi *et al.* injected adjuvant subcutaneously in the posterior feet [39]. Whether a subcutaneous posterior

foot injection may have caused tissue stress and the release of higher amounts of DNA from dying host cells, as observed with aluminum [14], triggering hypersensitivity reactions, toxicity and/or higher stimulation than occurs with intraperitoneal route immunization, remains to be evaluated.

The notably earlier onset and higher amount of proteinuria observed in the IFA as compared to the CFA group may be explained by considering the two different cytokine profiles likely induced by the two adjuvants: CFA has been reported to induce a significantly higher production of IL-17, IL-6 and TGF-β, while IFA appears to enhance IL-10 production [48, 49]; this cytokine is known to be involved in auto-Ab production and to contribute to the pathogenesis of SLE [50, 51].

A weight increase post-immunization has been previously reported by Oscherwitz *et al* in a non-autoimmune C57BL/6 mouse strain [44]. The observed increase was attributed to adjuvant-induced edema and/or inflammation, which may promote water retention [44]. Indeed, these investigators demonstrated that IFA and CFA were equally effective, and more so than ALU, in generating a mouse weight increase. In fact, while no differences were detected between the IFA and CFA treated groups, these mice showed a significantly higher weight than the mice in the ALU group [44, 52]. Thus, in agreement with these previous findings, IFA and CFA-treated mice displayed no weight differences, and weighed more than the mice in the ALU-treated group. At variance with the observations by Oscherwitz *et al*., we found no statistically significant difference between the weight of mice in the IFA or CFA groups as compared to the negative control group. Whether the discrepancy between Oscherwitz's and our results reflects the different strains of mice involved in the study, and hence a different susceptibility to immunization and/or bleeding stress, remains to be determined. A salient aspect of this analysis was the divergence between the weight differences observed in the early post-immunization period (from the 18^{th} to the 22^{nd} wk), and

the lack of differences in the middle post-immunization period (from the 23rd up to the 29th wk). By contrast, in the late post-immunization period (from the 29th to the 31st wk), an impressive and progressive loss of weight by almost one half was observed in 2 out of 3 ALU-treated mice, along with the appearance of a cachexia-like syndrome. An ALU-induced neurotoxic effect [53] was excluded as a possible cause of this syndrome since the mice continued to be mobile. It was also unlikely that ALU-induced peritoneal adhesions might have affected the gastrointestinal tract, inducing a malabsorption syndrome, since post-mortem observation of the peritoneal cavity showed fewer adhesions in the ALU as compared to the IFA and CFA-treated mice (data not shown). Finally, nephropathy was ruled out as a possible cause of this syndrome because it was not supported by proteinuria, which showed a similar progress to that observed in UNT mice.

Caution must be exercised in extrapolating the observed effect of adjuvants to the clinical setting in humans, considering: i) that the "adjuvant quantity/host's weight" ratio in this study was markedly higher than that in human vaccination; and ii) the different route of immunization employed, in that adjuvant was injected intraperitoneally in this study while the intramuscular route is commonly used for vaccination in human.

In conclusion, even if the power of the study is limited by the relatively low number of mice in each group, our data indicate that SQU seems to be a less toxic adjuvant as compared to ALU, did not accelerate proteinuria, as observed in the IFA-treated group, and did not elicit anti-RNP/Sm auto-Ab, as observed in the CFA group.

CONFLICT OF INTEREST.

YS declares that he has appeared in court as an expert witness in cases of subjects who developed autoimmune phenomena following vaccinations or silicone implants. All the remaining authors declare no conflicts of interest.

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FP, YS and EF conceived and designed the study; EF and FP drafted the manuscript. YS and FP participated in critical revision of the manuscript for important intellectual content. EF, EIF and LD carried out the immunoassays and acquired the data. EIF, LD and VR participated in the analysis and interpretation of data. EIF and VR performed statistical analyses. All authors read, revised and approved the final version of the manuscript. This work was supported by a grant in 2012 from "SLE Italian Group". The authors are grateful to Mr. Vito Iacovizzi for his excellent secretarial assistance.

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Table 1. Adjuvants most commonly employed in vaccine for human use.

Adjuvant name	Adjuvant class	Disease (vaccines trade name®)
Aluminium hydroxide	Mineral salts	Diphtheria, Tetanus, Pertussis, Haemophilus influentiae B, Hepatitis A/B, human papilloma virus, Anthrax.
Incomplete Freund's adjuvant	Mineral oil-in-water emulsion	Influenza, Experimental HIV, tumor
Squalene-based (MF59®)	Organic oil-in-water emulsion	Seasonal influenza (Fluad®), pandemic influenza (Focetria®), pre-pandemic influenza (Aflunov®)

LEGEND TO FIGURES

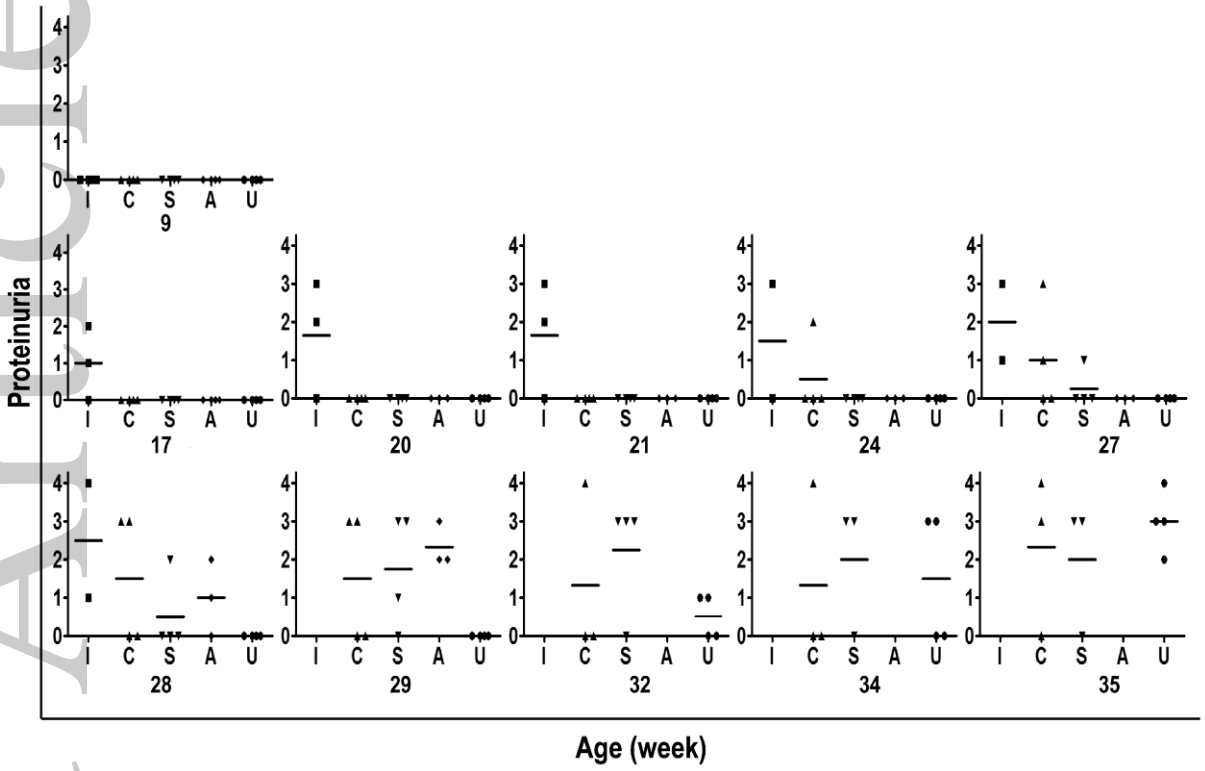

Fig. 1. Time course of mouse proteinuria.

Urine samples from individual adjuvant-treated and untreated mice were collected weekly, and proteinuria was assessed and rated on a 0–4 scale (0=15-20mg/dl; 1≥30 mg/dl; 2≥100 mg/dl; 3≥300 mg/dl; 4≥2000 mg/dl). Scatter plots showing the proteinuria distribution and mean value in each group at different time points. **I**: incomplete Freund's adjuvant; **C**: complete Freund's adjuvant; **S**: Squalene; **A**: aluminum hydroxide; **U**: adjuvant-untreated mice.

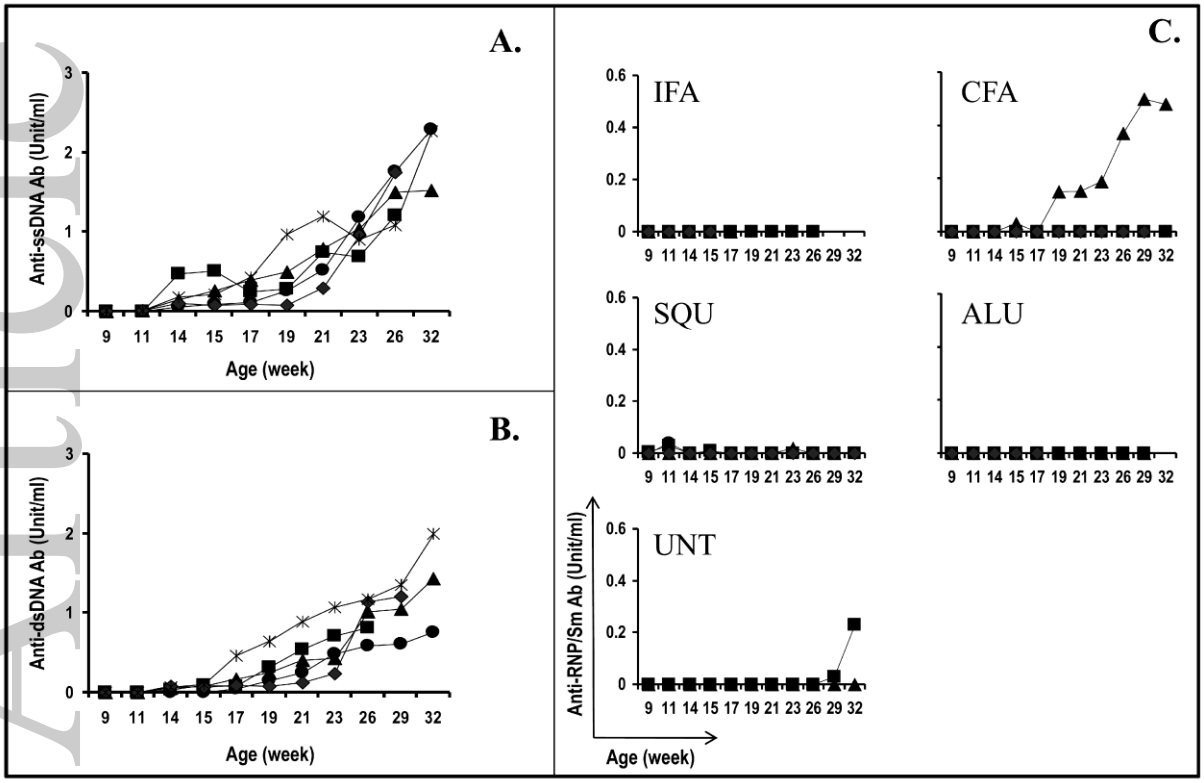

Fig. 2. Mouse anti-ssDNA and anti-dsDNA Ab during the course of the study.

(A., B) Sera (diluted 1:200 in PBS) from IFA- (■),CFA- (▲), SQU- (●), ALU-(♦) treated mice and untreated mice (⋆) were collected at the indicated time points and analyzed for the presence of anti-ssDNA Ab (**A.**) and anti-dsDNA Ab (**B.**). Each data point is the mean antibody titer in that group. (**C.**) The same sera were tested for the presence of anti-RNP/Sm Ab which are shown for individual mouse (each denoted by a symbol) in each group. Results in each panel are expressed as units/ml extrapolated from a calibration curve constructed with positive control serum. Data are representative of 2 different experiments.

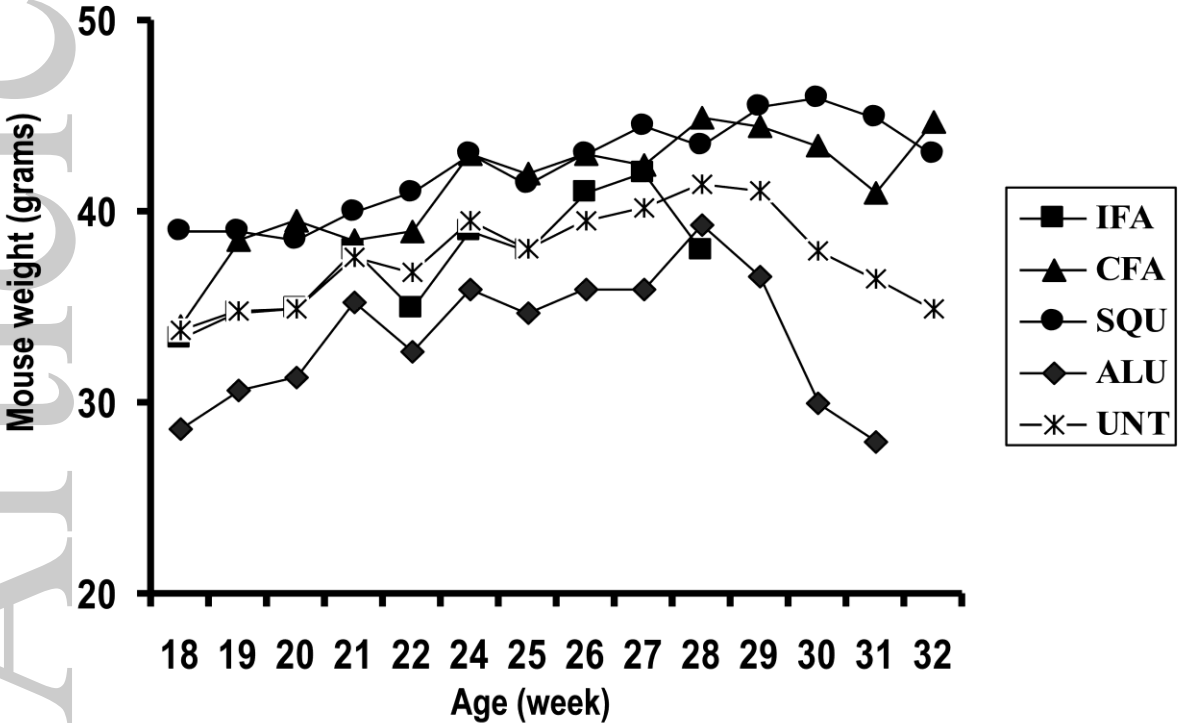

Fig.3. Mouse weight during the course of the study.

Individual IFA- (■),CFA- (▲), SQU- (●), ALU-(♦) treated mice and untreated (*) mice were weighed weekly starting from the 18th wk. Each data point represents the mean mouse weight in that group at the indicated wk.

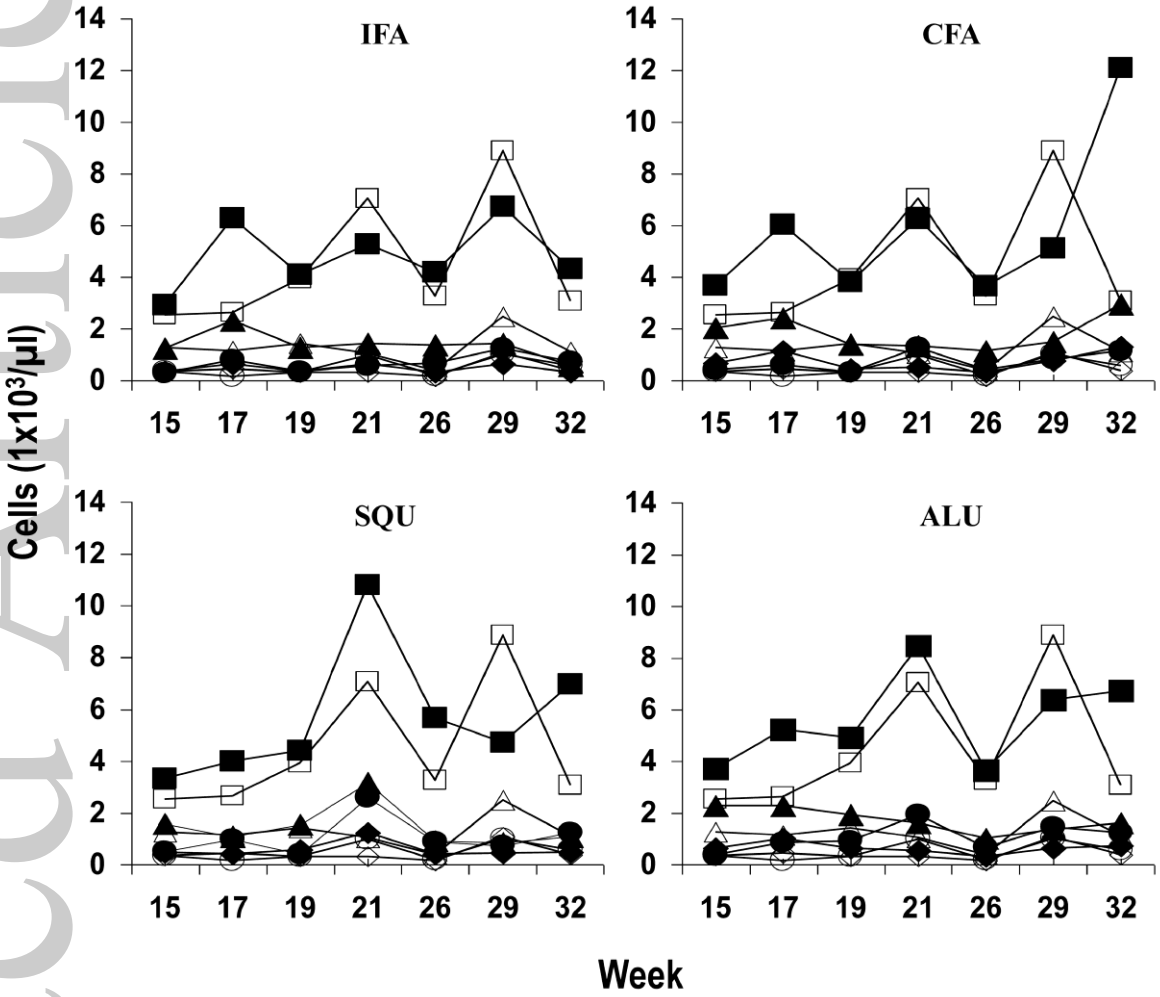

Fig. 4. Mouse peripheral blood mononuclear cell (PBMC) counts during the course of the study.

PBMC were obtained by Ficoll-Hypaque density gradient centrifugation of whole blood drawn from adjuvant-treated mice (closed symbols), and counted. Flow cytometry analysis of total lymphocytes (square symbol) and CD19+ (round symbol), CD8+ (rhombus symbol), CD4+ (triangle symbol) lymphocyte subsets was performed to determine the percentage of each PBMC subset. Subset count was obtained by multiplying the total lymphocyte number by the percentage of each cell subset. The mean subset count is shown for each adjuvant – treated group at each time point. Data from the untreated mouse group (open symbols) are reported in each panel for direct comparison.