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STUDIES OF ALTERED RESPONSE TO INFECTION INDUCED BY SEVERE INJURY

ANNUAL PROGRESS REPORT

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In this fourth year of the contract, 23 patients were assessed for altered monocyte immune functions. New assays were introduced to assess patient monocyte (MØ) production of Transforming Growth Factor-β (TGF-β) and Interleukin-6 (IL-6). Both MØ TGF-β and IL-6 production were found to be elevated in immunocompromised patients' MØ. Elevated TGF-β levels were demonstrated to further augment patient MØ Prostaglandin E_2 (PGE_2) production by an autocrine stimulation pathway. Elevated MØ IL-6 production was linked to the increased stimulation of patients' MØ Tumor Necrosis Factor-α (TNF-α) and to elevated MØ PGE_2 levels. We demonstrated that post-trauma increased MØ IL-6 would elevate immunoglobulin levels and thereby trigger increased stimulation of the patients' MØ 72kd receptor for immunoglobulin. This IL-6 stimulation of the MØ receptor results in elevated TNF-α and PGE_2. The therapeutic potential of synthetic glucans and the T cell product, Interleukin 4 (IL-4), were investigated. Addition of IL-4 to MØ was found to decrease
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trauma patients' elevated MØ production of IL-6, PGE_2, and TNF_α, even in cases where patients' MØ were already massively preactivated.

Some of the synthetic glucans were shown to downregulate MØ PGE_2 production without concomitant upregulation of patients' MØ TNF_α levels. Patients' MØ IL-6 production was unaffected or only slightly increased after glucan treatment. These in vitro, glucan results on patients' MØ confirm some preliminary animal reports that have appeared.

In summary, the septic syndrome was further defined in trauma patients by experiments defining the pathological role of increased TGF_β and IL-6. Two possible prophylactic agents, synthetic glucans and IL-4, were characterized and described.

FOREWORD

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Carol L Miller Burgess 8-21-90

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INTRODUCTION

The goal of this contract project is twofold: first to develop new assays for the assessment of immune aberrations after combat injury and, second to analyze immunomodulators for their potential in prophylactic therapy for reducing or moderating immune aberrations. The development of new monitoring methods involves determining the predictive value of the assay, adapting the assay for use with patient samples and assessing the suitability of these assays for use in far forward locations to monitor combat casualties. In order to evaluate various immunomodulators, it is necessary to characterize those immune aberrations which are pivotal in development of immunosuppression and/or which are pivotal in the pathology of septic organ failure and death. These pivotal aberrations can then be targeted for immunomodulatory therapy. This laboratory has focused both on changes in monocyte functions which lead to immunosuppression or pathology and on interactions between the trauma induced monokine alterations and increased or decreased monocyte functions which initiate or perpetuate post-trauma immunosuppression and immunopathology. The experiments have focused on assessing monocyte production of monokines and characterizing the casual mechanisms for these monocyte aberrations. Our data indicate that trauma induced shifts in the proportion of distinct MØ subsets account for a large portion of the MØ aberrations. These shifts result in the increase in some mediators and decrease of other mediators altering monocyte interaction with other immune cells. In particular, increases in MØ production of immunosuppressive PGE_2 can be related to increased proportion of one of the MØ subsets. However, other monokines may also be mediating immunopathology and related to MØ subset shifts. These relationships were the focus of this year's contract period.

METHODS

Peripheral blood MØ are separated by selective adherence from trauma and burn patients, as previously described (1). MØ from normal controls are separated along with the patients in each experiment. MØ plasminogen activator (PA), procoagulant activity (PCA) and lysozyme assays are performed as previously described (1). MØ antigen presentation capacity was tested as described by Goeken et al (2). MØ PGE_2 is measured in the ELISA assay, which was adopted in our laboratory during the previous contract year. MØ TNF levels were determined in the cell-free MØ supernates (secreted TNF) and in the sonicated MØ lysates (cell-associated TNF) using the L-M cell bioassay as previously described (3). Secreted IL-1 activity was measured in MØ supernates. The IL-1 specific D10.G4.1 cells were cultured in the presence of different dilutions of IL-1 containing MØ supernates. 2.5μg/ml Con A was used as a co-mitogen in RPMI media containing $5x10^{-5}$ M 2ME and 5% FBS. The proliferation of $2x10^4$ D10.G4.1 cells/well was measured using a ^{3}H-Thymidine pulse for the last 18 hours of the 72 hours proliferation assay. Sample activity was calculated by comparing the dilution of the samples to the dilution of the IL-1 standard resulting in 50% maximal proliferation. MØ IL-6 production was measured in the MØ supernates as previously described, using the B9 hybridoma cell line, which is highly specific for IL-6 as previously described (4). Briefly, $2x10^4$ B9 cells were seeded in serial dilutions of the MØ supernate samples or IL-6 standard on a 96 well plate. B9 proliferation was measured by 3H-TdR incorporation during the last 18 hours of the 96 hours proliferation assay. Activity of the samples was calculated by a computer program using our Compaq computer, using the following formula:

$$\text{Sample activity} = \text{Stand. activity} \times 2^{\text{Sample EP-St.EP}}$$

The Mv1Lu (mink lung) cell assay was utilized to measure the TGF_β production by trauma patients' and normal monocytes. Proliferation of the Mv1Lu cells is inhibited by TGF_β. TGF_β activity produced by MØ is in a latent form in the MØ supernates, because active TGF_β is complexed with a TGF_β binding protein, which is biologically inactive. TGF_β can be converted to its free, biologically active form with acid treatment. Therefore, our MØ supernate samples are acid treated (pH 2.5-2.8) for 2 hours. Their pH is then adjusted back to pH 7.32, filtered, and used for TGF_β determination in the Mv1Lu bioassay. Activity of the MØ supernates is calculated for the dilution of the sample and the standard resulting in half maximal inhibitors by the following formula:

$$\text{Sample activity: Std. Activity} \times 2^{\text{Sample EP-St. EP}}$$

MØ are isolated from each patient as early as 1-3 days post-injury, and blood is collected biweekly during their hospitalization. Each patient's blood sample is processed along with a normal's control blood donated by the research and hospital staff at the UMMC. MØ were stimulated with 20 ug/ml muramyldipeptide (MDP) alone, with a combination of 10^{-6} M Indomethacin (INDO) + MDP or with a suboptimal dose of Interferon gamma (IFN_γ) (20 mg/ml) plus 20 μg/ml MDP. In some experiments MØ were primed for four hours with 0.05-50 ng/ml IL-4 followed by 20 μg/ml MDP stimulation. The optimal concentration of IL-4 was 5 ng/ml, which has been used in the subsequent assays.

In certain experiments, particulate glucans were employed for MØ stimulation. Particulate glucan B and R4 was used in a concentration range of 0.1-50 μg/ml and 15 μg/ml was the optimal dose. Particulate glucan D served for structural glucan control, without biological effect. MØ supernates were collected after 16-20 hours incubation and the adherent MØ were collected with

short EDTA treatment followed by scraping. MØ were used for cell-associated TNF And PCA determinations after freezing-thawing and sonication.

MØ subsets are separated on the basis of the density of their high affinity Fc receptors for human IgG_1 and IgG_3 (FcRI) by rosetting the MØ with anti Rh-coated erythrocytes. Subset experiments are performed on trauma and burn patients to delineate injury induced changes in the ratios of MØ subpopulations determined by MØ surface markers. Subset experiments also attempt to investigate any differential monokine producing capacity or any differential stimulation requirements for the FcRI MØ subset. The cyclo-oxygenase inhibitor, Indomethacin (Indo) was used to inhibit MØ PGE_2 production. The effect of stimulation with Indo in combination with MDP was studied for MØ TNF induction. IL-4 alone or in combination with Indo + MDP was also utilized for MØ stimulation.

For determination of the percentage of MØ with different surface markers in the adherence separated MØ of post-trauma patients and normal controls, we increasingly utilized our newly obtained Epics FACS Analyzer. Direct fluorescence staining with FITC-labelled monoclonal antibodies is performed in our assays. FITC-labelled, matching type of mouse IgG control is always included for the determination of non-specific binding. Briefly, $1x10^6$ MØ are stained with the appropriate test-amounts of FITC-labelled OKM5, MY4, CD8, T3, FcRI or FcRII monoclonal antibodies, respectively. After 30 minutes incubation at 4°C, cells are washed three times to remove the excess antibody and resuspended in 1.0 ml resuspension media. Then, the cells are analyzed for fluorescence intensity by the previously established analysis program on the Epics. The percentage of positive MØ for each surface marker are compared between normal and patient MØ.

The levels of MØ monokine production may vary from individual to individual, with some high and some low responders. Consequently, Wilcoxon Signed Rank and Mann-Whitney U nonparametric statistics were utilized when appropriate to compare changes in MØ responses after different stimulation requirements from an individual blood donor as well as to compare the corresponding MØ response levels in post-trauma patients and normal controls. MØ responses in the patients are correlated to the clinical outcome and to the days in the post-injury period.

RESULTS AND DISCUSSION

During the research period covered by the fourth year of Contract No. DAMD A-86-C-6091, several new pieces of information have been attained in the area of our main goals: 1) the development of assays to monitor the development and progression of immunosuppression in post-trauma patients and 2) the investigation of possible prophylactic modalities to reverse or reduce post-trauma immune aberrations. During this year we have monitored our patients for levels of MØ Transforming Growth Factor$_\beta$ (TGF_β) and Interleukin-6 (IL-6). We have examined Interleukin 4 and Glucans as possible prophylactic agents. In addition, we have investigated and further characterized the mechanisms by which elevated immunosuppressed levels of PGE_2 are maintained in post-trauma patients.

Twenty-three patients have been monitored this year including 12 burn and 11 trauma patients. Of these patients, 4 were studied in the first three months, 6 patients during the second quarter, 5 patients during the third quarter and 8 patients in the last three months of the year. Of 23 participating patients, 5 succumbed to fatal sepsis. Out of the total 23 patients studied, characteristic MØ abnormalities are summarized for those 13 patients (8 trauma, 5 burn) who experienced septic episodes and immunosup-

pression (Table I). Markedly decreased mitogen induced mononuclear cell proliferation was observed in those patients with septic episodes. Decreased mononuclear cell PHA responses are a well-established and easily measurable parameter with a high predictive value for determination of the development of immunosuppression in trauma and burn victims. Consequently, we utilized this parameter to identify immunosuppressed patients. In addition, massively elevated MØ PGE_2 levels were found in immunosuppressed patients which occurred concomitant to decreased MØ plasminogen activator values. Both hyper-elevated PGE_2 and abnormal PA levels have been previously described by our laboratory and other investigators as characteristics of post-trauma patient's monocytes. We have recently described an additional aberration in post-trauma monokine production, dramatically increased MØ TNF production.

Our present data demonstrate that monitoring MØ TNF levels could have predictive value for post-trauma immunosuppression. Hyperelevated MØ TNF, particularly cell-associated MØ TNF, was found only in immunosuppressed patients, while MØ TNF levels of immunocompetent patients were only moderately elevated (Table II). Concomitant elevations of PGE_2 and TNF levels in monocytes of immunosuppressed patients suggests that regulation of monokine production in post-trauma MØ is different from that of normal MØ. High levels of PGE_2 have been shown to downregulate TNF production in normal MØ. In contrast, we found that TNF production in post-trauma patient's MØ is not sensitive to downregulation by excessive amounts of PGE_2. Our laboratory and several other investigators demonstrated that PGE_2 is one of the major mediators of post-trauma immunosuppression. Hyper-elevated MØ PGE_2 levels have inhibitory potential for a number of T cell functions, such as IL-2 receptor expression, T cell proliferation, etc. Production of IL-1 is also inhibited by high levels of PGE_2 in post-trauma patient's MØ. During the previous years of this contract, we demonstrated that high MØ PGE_2 levels inhibited MØ plasminogen activator expression. Plasminogen activator production by MØ has been implicated in the processing of MØ IL-1 and its depression correlates to depressed T cell activation. We also have data suggesting that post-trauma elevated MØ PGE_2 inhibits MØ-T cell interactions in the tetanus toxoid antigen presentation system.

We have also previously demonstrated that immunosuppressed post-trauma patients exhibited a shift in the relative proportion of their MØ bearing the high affinity IgG receptor, FcRI. We have also shown that the post-trauma monocyte aberrations can be correlated to functional differences between the FcRI expressing ($FcRI^+$) and FcRI non-expressing ($FcRI^-$) MØ subpopulation and increased numbers of $FcRI^+$ subpopulation post-injury. The monocyte subpopulation expressing high densities for the FcRI receptor ($FcRI^+$) produces high levels of MØ PGE_2 and has lower capacity for antigen presentation (APC). In contrast, the low density FcRI bearing, or FcRI negative ($FcRI^-$) MØ subpopulation has greater antigen presentation capacity concomitant to significantly lower MØ PGE_2 levels ($p<0.001$) (Fig. 1, Table III). Many of the post-trauma monocyte aberrations can be related to the increased numbers of the high PGE_2 producing FcRI MØ population. One could assume that the high PGE_2 levels in the $FcRI^+$ MØ are also responsible for the decreased antigen presenting capacity of this MØ population. However, when a cyclo-oxygenase inhibitor (Indomethacin) was added to the $FcRI^+$ MØ population to inhibit PGE_2 synthesis, the antigen presentation capacity of this MØ subset was still well below that of the $FcRI^-$ MØ subpopulation. These data suggested that although PGE_2 is probably one of the key monokines with negative regulatory effects in post-trauma immunosuppression, other monocyte-derived mediators might also be involved. One of these, tranforming growth $factor_\beta$, is discussed below.

We made significant progress this year in monitoring the complex interactions of cytokines in inducing altered MØ functions post-trauma. One such cytokine, Interleukin 6, has been implicated in the production of acute phase reactants and in mediating the metabolic derangement typical of septic trauma patients. We improved our B9 cell bioassay for determination of MØ interleukin 6 (IL-6) levels. IL-6 is primarily produced by MØ as well as by other cell types. Elevated levels of serum and urinary IL-6 have been recently reported after burn and elective surgery. Our data, gathered during this contract year, demonstrate that monocytes are the most likely source of the post-injury elevations serum IL-6 levels (Fig. 2). Monitoring MØ IL-6 levels from 16 trauma and burn patients, we found significant elevations in the MØ IL-6 levels of immunocompromised patients. MØ IL-6 levels of the immunocompetent patients were not significantly elevated compared to the normals. Elevations in MØ IL-6 levels occurred concomitant to septic episodes in immunocompromised patients (Fig. 3), suggesting that IL-6 is a valuable parameter for monitoring both immune status and determining risk of hepatic metabolic alterations of post-trauma patients. We have also characterized patients' MØ IL-6 responses to a variety of stimulators including FcRI crosslinking, MDP, the classical combination of IFN$_\gamma$ plus MDP as well as cyclooxygenase inhibitor plus MDP. Again we are assessing hyper/hyporesponsiveness of IL-6 induction to a variety of stimuli known to be present in the post-trauma environment. Stimulation with the bacterial analogue MDP significantly increased the MØ IL-6 levels in MØ of post-trauma patients ($p<0.04$). In addition, the MDP induced patient MØ IL-6 levels were significantly greater than MDP induced IL-6 levels in normal MØ ($p<0.03$). These results further support the idea of <u>in vivo</u> MØ preactivation by the post-trauma mediator environment.

One of our novel findings during this contract year was that IL-6 can be induced by FcRI receptor crosslinking/stimulation both in patient's and normal's MØ. MØ stimulation through FcRI has been shown to induce PGE_2, TNF and procoagulant activity by our laboratory and other investigators. MØ Fc-stimulation has clinical relevance since this type of stimulation would occur in post-trauma patients with circulating antibodies and sepsis or bacterimia. In addition, we have previously shown that immunosuppressed post-trauma patients experience a shift to increased numbers and proportion of the MØ subpopulation which expresses high densities for FcRI ($FcRI^+$) at the expense of the FcRI negative MØ subpopulation. The greater PGE_2 and TNF production by the $FcRI^+$ and the increased proportion of this MØ subset has been correlated to the elevated MØ PGE_2 and TNF levels in post-trauma MØ. We demonstrated during the present contract year that the FcRI positive MØ can be induced by crosslinking the FcRI (rosetting) to produce significantly higher levels of IL-6 production than can the FcRI negative MØ (Fig. 4). Higher FcRI stimulated IL-6 levels were found both in post-trauma patients ($p<0.001$) and normals ($p<0.001$) $FcRI^+$ MØ subset. In addition, FcRI stimulation augmented the levels of MØ IL-6 production in response to subsequent bacterial stimulation in the patient's $FcRI^+$ MØ subset ($p<0.02$) Elevated IL-6 levels in the immunosuppressed post-trauma patients $FcRI^+$ MØ subpopulation occurred concomitant to an increase in the proportion of their $FcRI^+$ MØ. As can be seen in Fig. 5, the aberrant levels of IL-6 correlated to an increase in the relative proportion of the patients' FcRI MØ subset.

This finding is further evidence to confirm our previous postulate that the increased number and ratio of the $FcRI^+$ MØ subpopulation in immunosuppressed post-trauma patients can be at least partially responsible for the altered monokine levels post-trauma. Consequently, monitoring patient's $FcRI^+/FcRI^-$ MØ ratios can be utilized as an indicator of aberrant monckine activities and

immunosuppression. Monocyte TNF, IL-6 and PGE_2 levels are elevated in trauma patients and the $FcRI^+$ MØ subpopulation produces significantly greater levels of these monokines than does the FcRI MØ subset. These results indicate again that screening of post-trauma victims for a shifted MØ FcRI ratio (increased $FcRI^+$ MØ ratio) can be a diagnostic tool for predicting the development of post-trauma immunosuppression and/or immunoaberrations. Induction of the $FcRI^+$ MØ subset caused concomitant elevation in PGE_2 and IL-6 production. MØ PGE_2 inhibits production of many monokines and downregulates a number of immune functions. Consequently, we assessed the relationship between MØ IL-6 and PGE_2 production in post-trauma patients. The concomitant elevation of MØ IL-6 and PGE_2 observed in the post-injury patients indicates that MØ IL-6 is probably not downregulated by high PGE_2 levels (Fig. 6). However, MØ IL-6 inhibition by extremely high MØ PGE_2 levels cannot be ruled out because PGE_2 levels >30 ng/ml did not occur in the assays evaluated.

The above pattern of MØ IL-6 independence from PGE_2 regulation is similar to our previous observation of PGE_2 effects on MØ TNF responses in immunosuppressed post-trauma patients. Although IL-6 and TNF production does not seem to be downregulated by massively elevated PGE_2 levels in post-injury immunosuppressed patient's MØ, PGE_2 is one of the pivotal mediators of other types of post-trauma MØ immunosuppression. However, we have also shown that patient's MØ depression in PA activity and APC cannot be totally explained by elevated PGE_2 production.

Transforming growth factor beta (TGF_β) is another inhibitory mediator produced by MØ which is being assessed in our laboratory. Last year we initiated the TGF_β assay system and we have made significant progress this year in characterizing the levels of TGF_β production by post-injury patient's MØ. TGF_β is a known inhibitor of T cell proliferation and other immune functions, including production of certain monokines such as PA. Therefore, we examined the TGF_β production of patients' and normals' MØ after adherence isolation and bacterial stimulation. MØ supernates from 11 immunocompetent and 17 immunosuppressed (immunocompromised) patients were assessed for TGF_β after no other stimulation but adherence isolation. Immunocompromised patients' MØ TGF_β levels were significantly higher than the TGF_β levels of MØ from immunocompetent patients or normals (Table IV). TGF_β levels of MØ from immunocompetent post-injury patients were in the range of those from normals' MØ (Fig. 7). When trauma patients' MØ were stimulated with a bacterial product analogue, muramyl dipeptide (MDP), markedly greater TGF_β levels were stimulated in the patients' MØ than in normals' MØ. In addition, unstimulated immunosuppressed patient's MØ had significantly elevated TGF_β production. These data again suggest that immunocompromised post-trauma patients' MØ are pre-activated by their post-trauma microenvironment to produce aberrant monokine levels and consequently also have greater susceptibility to subsequent bacterial stimulation. However, addition of interferon gamma (IFN_γ, 100 U/ml) could decrease in some cases the MDP induced MØ TGF_β levels both in normal and trauma patients' MØ (Fig. 8). This immunomodulator effect of IFN_γ is being further assessed. Elevated levels of MØ TGF_β and PGE_2 occurred concomitantly in the immunosuppressed patients. Addition of cyclo-oxygenase inhibitor did not increase or decrease MDP induced MØ TGF_β levels, suggesting that production of TGF_β and PGE_2 at the monocyte level is independent.

In summary, the increased TGF_β production by post-injury monocytes that we have detected further implicate the critical role of monocyte mediators in the development of post-trauma immunosuppression. We have shown that elevated MØ TGF_β production can also inhibit the overall immunological responses contributing to patient's immunosuppressed status in addition to the

immunosuppressed effects of elevated PGE_2.

During this contract period, we made further progress in understanding some of the regulatory mechanisms resulting in the hyper-elevated PGE_2 and TNF levels in post-trauma patient's MØ. Since MØ TGF_β levels increased concomitant to increased MØ PGE_2 production, we examined TGF_β for induction of PGE_2 in MØ. We showed that TGF_β is a potent inducer of normal MØ PGE_2 (Fig. 9). These data imply that post-trauma elevations in MØ TGF_β would further potentiate post-trauma immunosuppression by inducing/increasing MØ PGE_2 levels. TGF_β has been shown to decrease MØ TNF bioactivity while increasing the mRNA levels for TNF. We found that although TGF_β decreases the levels of secreted normal MØ TNF, the cell-associated TNF levels were increased (Fig. 10). This redistribution of MØ TNF (more cell associated TNF than secreted TNF) in response to TGF_β is similar to the aberrant TNF pattern seen in immunosuppressed, post-trauma, patient's MØ. Our data from the last year of this contract demonstrated a unique pattern of hyper-elevated cell-associated MØ TNF in immunosuppressed patients. Consequently, our TGF_β data suggest that elevated MØ TGF_β levels in the post-trauma patients might contribute to the abnormal elevations of MØ TNF, particularly MØ cell-associated TNF production. These data also imply that TGF_β has regulatory properties in the post-trauma immune system. Consequently, monitoring of MØ TBF_β can be predictive of the development of the immunocompromised state in post-injury patients. We are actively investigating the ability of TGF_β to redistribute patient MØ TNF production toward the long-lived cell-associated form. Patient MØ are being evaluated for hyperesponsiveness to TGF_β effects.

During this contract year, we successfully expanded our experiments investigating the second specific aim. We evaluated Interleukin-4 and particulate glucans as possible prophylactic agents to reduce post-injury MØ aberrations. Interleukin-4 is a recently described and cloned lymphokine, which was originally described as a B-cell activating factor but which has regulatory effects on MØ. Downregulation of MØ normal peripheral blood monocytes IL-1, TNF_α and PGE_2 production by IL-4 has been reported by several investigators including our laboratory. During the last contract year, we also demonstrated that <u>in vitro</u> IL-4 treatment can decrease the extremely high MØ TNF_α levels in post-trauma patients. Furthermore, IL-4 particularly decreased the cell-associated TNF in the high PGE_2 producing $FcRI^+$ MØ subpopulation. Since the increased number and proportion of the greater TNF producing MØ $FcRI^+$ subset can account for the massive amounts of cell-associated MØ TNF typical of immunosuppressed trauma patients. In addition cell-associated TNF has been implicated in mediation of endotoxin shock. Consequently downregulation of total and cell-associated TNF by IL-4 has therapeutic implications for immunosuppressed post-injury patients. In addition, we have demonstrated that IL-4 can inhibit the highly elevated PGE_2 levels in patient's MØ. As illustrated in Fig. 11, IL-4 significantly downregulated the IL-6 levels both in the patient and normal MØ. The IL-4 inhibition was particularly impressive in the FcRI positive MØ, which produce greater levels of IL-6. Data from Table V indicate that IL-4 was also a potent inhibitor of MØ IL-6 levels whether MDP combination of IFN, Indomethacin plus MDP was the IL-6 inducing signal in the post-trauma patient's and normal MØ. However, downregulation of MØ IL-6 was concomitant to downregulation of MØ PGE_2 in the post-injury patients. IL-6 has several immunoregulatory and inflammatory activities. IL-6 induces acute phase reactants and it has also been implicated in the development of post-trauma metabolic changes. Consequently, IL-4 induced concomitant downregulation of the post-trauma elevated MØ IL-6, TNF and PGE_2 potentially could correct some of the post-trauma MØ aberrations.

The other group of immunomodulatory modalities we examined in this contract period are particulate glucans B and R4. These synthetic glucans are derived from saccharomyces but branched similarly to candida. The yeast product Zymozan is a potential MØ activator that has been described as preferentially activating lypoxygenase products instead of cyclo-oxygenase. Consequently, the synthesized yeast glucan B and R4 could also have differential regulatory effects on monokine production. Examination of the effect of glucans on immunosuppressed trauma patient's MØ had a twofold purpose. First, to investigate the patient's monokine production in response to yeast-like stimulation. Our second purpose is to determine if some yeast analogues preferentially increase certain monokines such as IL-1 without concomitantly increasing MØ PGE_2 or TNF levels. Yeast infections and septic episodes with fungal etiologic background are very common in immunocompromised patients. Since the chemical structure of the particulate glucans, as well as the soluble forms of the glucans investigated in our study has great similarity to that of the candida, we utilized these glucans for in vitro MØ stimulation.

Human monocytes possess a receptor for soluble and particulate glucans, the β-glucan receptors. Glucans have been shown to activate the alternative complement pathway, phagocytosis and leukotriene generation. Table VI illustrates PGE_2 data on normal MØ. Addition of particulate glucan B or R4 to MØ resulted in a dose-dependent inhibition of MØ PGE_2 production. Particulate glucan D, which was a control for the particulate structure had no effect on MØ PGE_2 levels. Particulate glucans B and R4 were also potent inhibitors of MØ PGE_2 production in post-injury patients with elevated MØ PGE_2 levels (Table VII). Furthermore, glucans were able to downregulate trauma patient's elevated MØ PGE_2 production in the face of bacterial MØ stimulation with MDP (Table VIII). These data might suggest that glucan analogues could modulate aberrant MØ functions post-trauma. Experiments utilizing soluble analogues of these particulate glucans need to be performed. In addition to their inhibitory effect on MØ PGE_2, particulate glucan B and R4 showed downregulating potential on MØ IL-6 production as well (Table IX). In contrast to thei anti-inflammatory-type effect on MØ IL-6 and PGE_2 production, particulate glucans B and R4 stimulated MØ TNF production in normals (Table X). Since MØ TNF is massively elevated in immunosuppressed trauma patients, further elevation of patient's MØ TNF levels would increase their risk for TNF mediated septic complications. However, our preliminary data indicate that low dose particulate glucan R4 might actually downregulate the elevated TNF levels in trauma patient MØ. Further experiments need to be done to investigate this effect. Nevertheless, our preliminary results on the effect of glucans on trauma patient's monokine production indicate that distinct glucan analogues with slightly different chemical structure might have different effects on the altered production on MØ TNF PGE_2, IL-6 etc. in trauma patients. Such a selective effect of glucan analogues would be particularly applicable in restoring of patient's MØ responses.

In summary, this contract year has been particularly productive. New assays for monitoring patient TGF_β and IL-6 have been introduced. Some of the mechanisms which lead to elevated PGE_2 and cell-associated TNF have been characterized. The possible immunotherapeutic roles of IL-4 and glucans has been explored. As we move into the final year, the role of leukotrienes, IL-8 and the IL-1 inhibitor will be examined leading to more data which is applicable to the care and treatment of combat casualties.

TABLE I

Correlation of PHA response, MØ PA, PGE_2 and TNF aberrations and clinical outcome

PATIENT	INJURY	PHA[a]	PA[b]	PGE_2[c]	TNF[d]	OUTCOME
1S	Burn	-71%	9.75(18.8)	7.9— 34.3	154.7(0.0)	Septic episodes
2F	Burn	-59%	24.8 (14.5)	4.4— 26.5	221.2(0.0)	Septis, expired
3Mc	Burn	-60%	ND[e]	22.1—156.1	80. (0.0)	Septic episodes
4G	Burn	-34%	10/14.8	7.5— 59.7	91.3(0.0)	Septic episodes
5La	Burn	-85%	ND[e]	0.7— 10.5[f]	5.7(0.0)	Septic
6Ha	Burn	-48%	12.9(39.6)	2.8— 5.3[f]	31.6(0.0)	Sepsis, expired
7La	Trauma	-82%	ND	16.3(6.2)	257.4(3.8)	Septic episodes
8Mf	Trauma	-51%	ND[e]	12.4(5.5)	17.8(0.0)	Septic episodes
9T	Trauma	-69%	ND[e]	31.1—132.2	52.4(24.8)	Septic episodes
10C	Trauma	-98%	ND	1.1— 93.8	53.7(0.0)	Septic episodes
11Ca	Trauma	-79%	10.3(17.3)	22.8— 60.1	280.2(0.0)	Septic episodes
12Sc	Trauma	-99%	ND[e]	3.9— 47.6	25.7(0.0)	Septic
13G	Trauma	-87%	22.6/19.0	0.3— 8.9[f]	4.4(0.0)	Sepsis, expired
14N	Trauma	-79%	5.7(19.0)	0.9— 11.4[f]	9.3(1.6)	Sepsis, expired
15Dy	Trauma	+0.05%	ND[e]	14.2— 28.6	79.4(0.9)	Sepsis, expired

[a] - Phytohemagglutinin response as decreases in % at 2μg/ml

[b] - Plasminogen activator in specific fibrinolytic units

[c] - Prostaglandin E_2 in ng/10^6 MØ

[d] - Tumor Necrosis Factor in ng/10^6 MØ

[e] - No Data

[f] - Expired before day 4 or no measurement on days of septic episodes

TABLE II

		MDP			INDOMETHACIN/MDP		
Patient	Days Post Injury	Secreted TNF[a]	Cell-Associated TNF[a]	PGE_2[b]	Secreted TNF	Cell-Associated TNF	PGE_2
Pt. 1	(1)	0	7.5	22.3	0	9.7	.241
Pt. 1	(3)	0	52.6	25.0	9.3	41.4	.135
Pt. 2	(3)	0	21.7	8.6	10.5	7.6	.172
Pt. 2	(10)	7.7	9.7	17.1	12.5	13.3	.103
Pt. 2	(12)	0	102.3	18.6	4.2	116.0	3.5
Pt. 2	(30)	0	5.2	6.5	0	9.1	.497
Pt. 3	(3)	0	61.1	4.8	0	65.8	.486
Pt. 3	(5)	0	37.4	1.1	0	40.7	.045
Pt. 3	(10)	4.0	14.1	33.7	3.1	16.6	1.2
Pt. 3	(19)	18.2	86.0	42.4	20.0	81.3	.121
Pt. 3	(26)	4.5	10.3	26.1	6.1	11.5	.9
Pt. 3	(31)	.6	6.7	50.4	.7	8.6	4.3
Pt. 3	(39)	1.6	3.6	3.7	.8	2.8	-
Pt. 3	(45)	3.7	5.4	93.5	5.9	9.5	1.2
Pt. 4	(6)	.6	2.6	97.9	1.6	4.8	.40
Pt. 4	(13)	0	239.2	53.6	0	256.1	.40
Pt. 5	(3)	0	0	33.8	.3	1.3	.30
Pt. 5	(8)	0	398.5	54.3	0	312.6	.32

a) TNF measured in the L-M bioassay and expressed as ng/10^6 MØ/ml.

b) Prostaglandin E_2 (PGE_2) measured in a sensitive ELISA assay and expressed as ng/10^6 MØ/ml.

TABLE IV

IMMUNOCOMPROMISED TRAUMA PATIENTS' MØ SHOWED INCREASED TGF_β LEVELS[2] WITH NO STIMULATION BUT ADHERENCE[3] ISOLATION

TGF_β in pM/10^6 MØ[4]	NORMAL CONTROLS' MØ	IMMUNO-COMPROMISED PATIENTS' MØ	IMMUNO-COMPETENT PATIENTS' MØ
	n = 20	n = 17	n = 11
range	0--19.1	24.7--415.4	0--17.2
median	0	101.8	0
mean + S.D	5.9 ± 7.9	148.4 ± 107.9	5.3 ± 7.2
signficance[5]		p< .003	N.S.

1. Immunocompromised patients = patients with depressed mitogen responses who subsequently experience septic episodes.
2. Transforming growth factor β (TGF_β) levels were determined in a mink lung bioassay.
3. Adhered to microexudate coated flasks.
4. TGF_β pM determined from recombinant TGF_β standard.
5. Significance determined in Wilcoxon test for paired nonparametric samples.

TABLE V

PERCENT REDUCTION OF IL-6 LEVELS BY IL-4 IN TRAUMA PATIENT'S MØ

IL-4 plus[c]:	Exp.#	NORMAL MØ[b]	NORMAL FcRI$^+$	NORMAL FcRI$^-$	PATIENT MØ	PATIENT FcRI$^+$	PATIENT FcRI$^-$
MDP	155	66	71	N.D.			
	157	57	74	70			
	159	78	83	92			
	175	57	63	42	71	68	20
	176	54	84	68	76	84	68
	178	99	75	75	60	64	78
	$\bar{x}$+S.D.	69±17	75±8	69±18	69±8	72±10	55±31
IFN_γ+MDP	200	54	36	13	46	28	1J
	204	42	21	20	41	43	37
	205	49	31	N.D.	20	80	5
	163	68	73	82			
	269		75	61	72	77	61
	166	73	69	69			
	178	89					
	$\bar{x}$+S.D.	63±17	51+24	49±31	45±21	57±25	29±25
Indo+MDP	163	56	72	70			
	166	78	69	67			
	188	61	71	52	28	79	22
	200	57	58	97			18
	269		75	68	72	77	61
	176	N.D.	74	N.D.	76	89	68
	178	89	62	100	70	50	79
	$\bar{x}$+S.D.	68±15	69±6	76±19	62±22	74±17	45±30

a) Percent reduction is calculated by the following formula:
$$\frac{\text{IL-6 in the presence of stimulus - IL-6 in the presence of stimulus + IL-4}}{\text{IL-6 in the presence of stimulus}}$$

b) IL-6 responses were measured in the 1) FcRI stimulated, FcRI positive MØ subpopulation and in the 2,3.) FcRI non-stimulated, whole MØ population and the FcRI negative MØ subpopulation.

c) IL-4 was used at 5 mg/ml/10^6 MØ concentration in a combination with 20 μg/ml MDP(MDP,), 10^{-6} M Indomethacin plus 20 μl/ml MDP (IFN_γ + MDP), respectively. The same stimuli were applied without IL-4 as positive controls. MØ were incubated with these stimuli for 16 hours.

TABLE VI

PROSTAGLANDIN E_2 (PGE_2) DOWNREGULATION BY B WGP AND R4 WGP ON NORMAL HUMAN MONOCYTES

Stimulation	Exp.#2	Exp.#4	Exp.#5	Exp.#6
medium	19.6	15.2	9.1	9.8
MDP 20ug/ml	23.6	21.4	N.D.	N.D.
B WGP 1ug/ml	N.D.	9.2	7.9	9.2
5ug/ml	21.3	10.1	4.5	7.2
15ug/ml	9.5	6.1	2.5	4.5
50ug/ml	2.5	N.D.	N.D.	N.D.
R4 WGP 1ug/ml	N.D.	5.8	6.1	12.6
5ug/ml	8.5	7.2	5.5	10.7
15ug/ml	5.7	2.9	2.5	6.0
50ug/ml	1.8	N.D.	N.D.	N.D.
D WGP 1ug/ml	N.D.	10.6	9.2	9.2
5ug/ml	14.2	10.2	7.2	9.3
15ug/ml	13.6	13.9	N.D.	9.0
50ug/ml	14.5	N.D.	N.D.	N.D.

a. PGE_2 is measured in the cell free monocyte supernates after 16 hours stimulation as indicated in a highly sensitive ELISA. PGE_2 levels are expressed as ng/10^6 monocytes/ml.

TABLE VII

Percent downregulation of MØ PGE_2 production by particulate glucans in trauma patients[a]

		Patients		Controls	
		B WGP[b]	R4 WGP[b]	B WGP	R4 WGP
Exp. 1	Pt. 1	65%	44%	89%	80%
	Pt. 2	77%	89%		
Exp. 2	Pt. 3	83%	69%	89%	78%
Exp. 3	Pt. 4	45%	39%	81%	34%
Exp. 4	Pt. 5	Not Done	45%	Not Done	89%
	Pt. 6	83%			

a. Percent downregulation of MØ PGE_2 is culculated by the following formula: percent downregulation = $\frac{PGE_2 \text{ in medium} - PGE_2 \text{ with glucan}}{PGE_2 \text{ in medium}}$

b. Particulate glucans B and R4 were used at 15μg/ml concentration.

TABLE VIII

Downregulation of MDP induced elevated PGE_2 levels by glucans in patients' monocytes[a]

	Patients		Normal	
	PGE_2 ng/ml	% downregulation	PGE_2 ng/ml	% downregulation
medium	4.80	-	16.23	-
MDP	33.75	-	15.85	-
MDP + B	7.54	78%	2.30	85%
MDP + R4	6.75	80%	3.12	80%
B WGP	3.01	37%	2.06	87%
R4 WGP	1.18	75%	1.91	88%

a. MØ PGE_2 levels were measured after stimulation with 20μg/ml MDP, 15μg/ml B WGP, 15μg/ml R4 WGP or with their combinations as indicated for 16 hours.

TABLE IX

Downregulation of monocyte Il-6 by B WGP and R4 WGP

Stimulation	Exp.#2.	Exp.#4.
medium	654	910
B WGP 1ug/ml	N.D.	506
5ug/ml	926	347
15ug/ml	559	240
50ug/ml	256	N.D.
R4 WGP 1ug/ml	N.D.	665
5ug/ml	391	574
15ug/ml	415	574
50ug/ml	211	N.D.

Il-6 is measured in the higly sensitive B9 cell assay. Il-6 activity is expresses as U/10^6 monocytes/ml.

TABLE X

TNF Induction By B WGP and R4 WGP in Normal Human Monocytes

Stimulation	Exp. #2	Exp. #4	Exp. #5	Exp. #6
medium	0/0 (0)[a]	0/0 (0)	0/0 (0)	0/0
IFN+MDP	69.0/0 (69.0)	0.8/1.4(2.2)	3.9/0.5(4.4)	1.3
B WGP 1ug/ml	- -	0.6/4.9(5.5)	0/0.6(0.6)	0
5ug/ml	16.3/14.1(30.4)	3.2/2.7(5.9)	27.5/1.7(29.3)	1.4
15ug/ml	31.1/10.9(42.0)	0.9/1.5(2.4)	43.8/1.3(45.1)	4.3
50ug/ml	22.7/9.0(31.7)	-	-	-
R4 WGP 1ug/ml	-	0/2.0(2.0)	0/0(0)	0
5ug/ml	9.1/10.3(19.4)	0.4/2.7(3.1)	1.2/0.9(2.1)	1.4
15ug/ml	19.0/10.8(29.9)	1.1/3.9(5.0)	12.8/0.3(13.1)	5.9
50ug/ml	7.9/8.8(16.7)	-	-	-
D WGP 1ug/ml	-	0/0(0)	0/0.8(0.8)	0
5ug/ml	4.4/0(4.4)	0/3.0(3.0)	0/0(0)	0
15ug/ml	4.8/0(4.8)	0.5/3.6(4.1)	0/0.5(0.5)	0
50ug/ml	3.5/0(3.5)	-	-	-

a) TNF is measured in the L-M cell bioassay. MØ TNF activity is expressed in ng/10^6 MØ/ml as follows: secreted TNF/cell-associated TNF. Total MØ TNF activity is shown in parenthesis. Secreted TNF is tested in the MØ supernates and cell-associated TNF is measured in the sonicated MØ lysates.

TABLE III

FcRI⁻ MØ Subset Has Greater Activity in Plasminogen Activator Production and Antigen Presentation Capacity

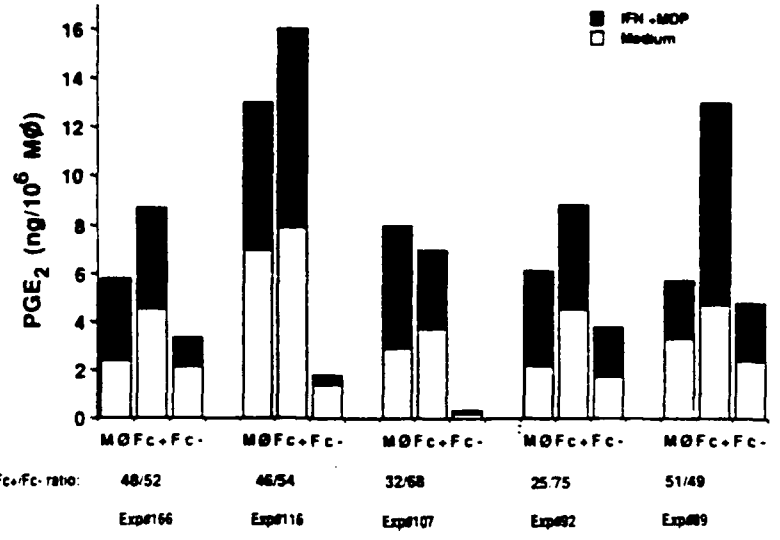
	Plasminogen activator (% specific fibrinolysis)[a]			Antigen presentation (cpm)		
	Exp. 1	Exp. 2	Exp. 3		Exp. 4	Exp. 5
FcRI⁺[b]	19.0	36.1	23.7	FcRI⁺[c]	9,165	10,437
FcRI⁻	57.2	51.4	35.5	FcRI⁻[c]	52,637	35,824

[a]MØ plasminogen activator production was measured as described in "Materials and Methods."

[b]FcRI⁺ and FcRI⁻ MØ subsets were separated by rosetting the MØ with anti-RH-coated human erythrocytes as described in "Materials and Methods."

[c]FcRI⁺ and FcRI⁻ MØ were pulsed with antigen (tetanus toxoid) in the antigen presentation assay. After the removal of excess antigen, antigen-pulsed MØ were co-cultured with syngeneic T cells.

FIGURE 1



Greater PGE_2 production by an FcRI⁺ MØ subset without and after stimulation. Equal number of both the MØ and FcRI MØ subsets were stimulated with a combination of IFN-γ (100 U/10^6 MØ) plus MDP (20 μg/ml), respectively. After 16 hours culture, PGE_2 was assayed in the MØ supernates by the ELISA method described in "Materials and Methods." MØ PGE_2 levels are shown as stack columns where MØ PGE_2 levels are shown on the bottom of the column, and the top of the column represents the PGE_2 levels produced by the MØ above the medium control level upon stimulation. PGE_2 levels of the FcRI⁺ MØ subset were statistically greater both unstimulated ($P < .001$) and after IFN-γ + MDP stimulation ($P < .001$) than that of the FcRI⁻ MØ subset as calculated from the total of 23 experiments. The ratios of the FcRI⁺ and FcRI⁻ MØ subsets (Fc^+/Fc^- ratio) within the unseparated MØ population (MØ) are shown for each experiment. Each experiment represents a different individual blood donor.

FIGURE 2

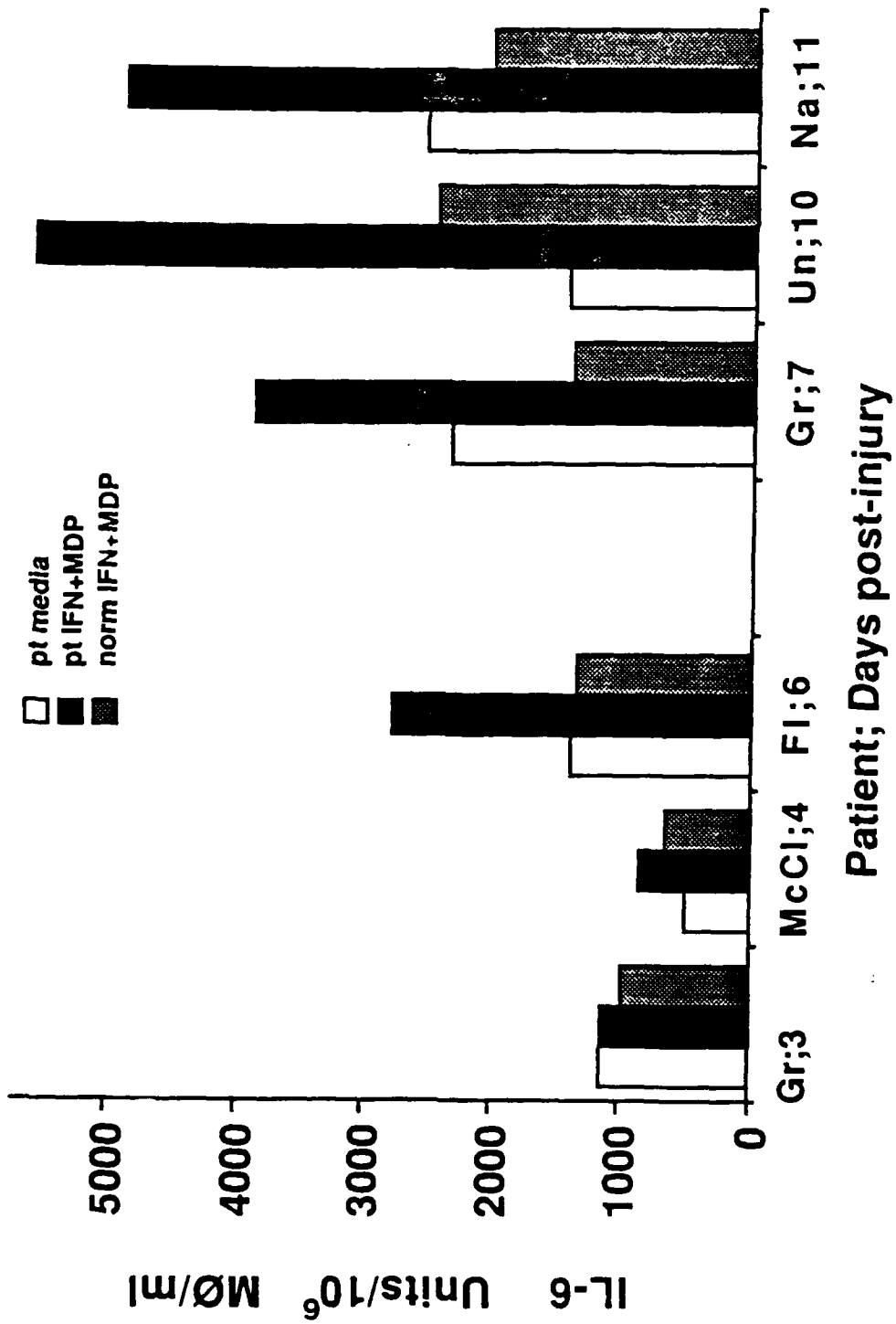

IL-6 Units/10^6 MØ/ml
5000
4000
3000
2000
1000
0
pt media
pt IFN+MDP
norm IFN+MDP
Gr;3
McCl;4
Fl;6
Gr;7
Un;10
Na;11
Patient; Days post-injury

FIGURE 3

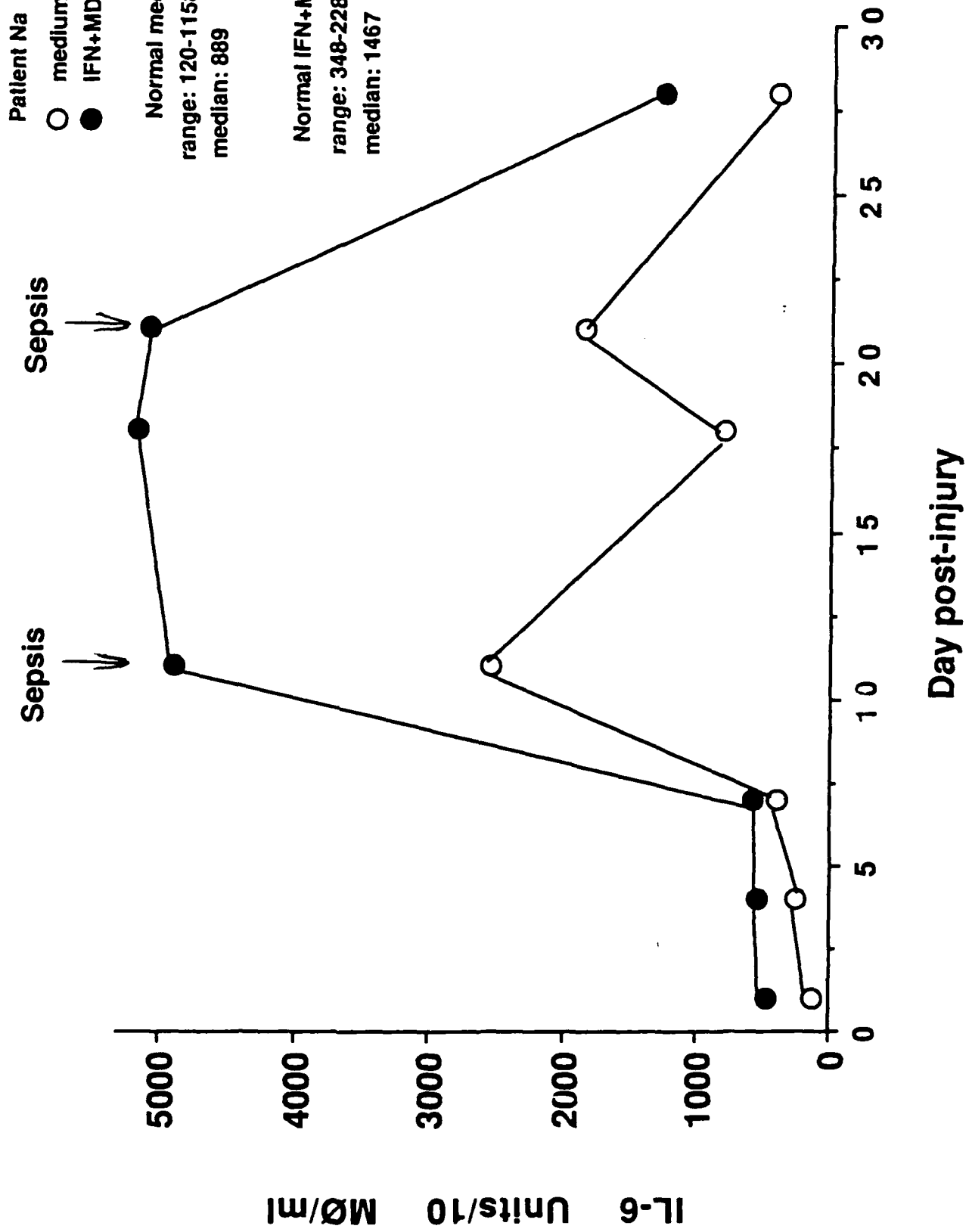

Patient Na
medium
IFN+MDP
Normal media:
range: 120-1158
median: 889
Normal IFN+MDP:
range: 348-2285
median: 1467
Sepsis
Sepsis
IL-6 Units/10 MØ/ml
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
Day post-injury

FIGURE 4

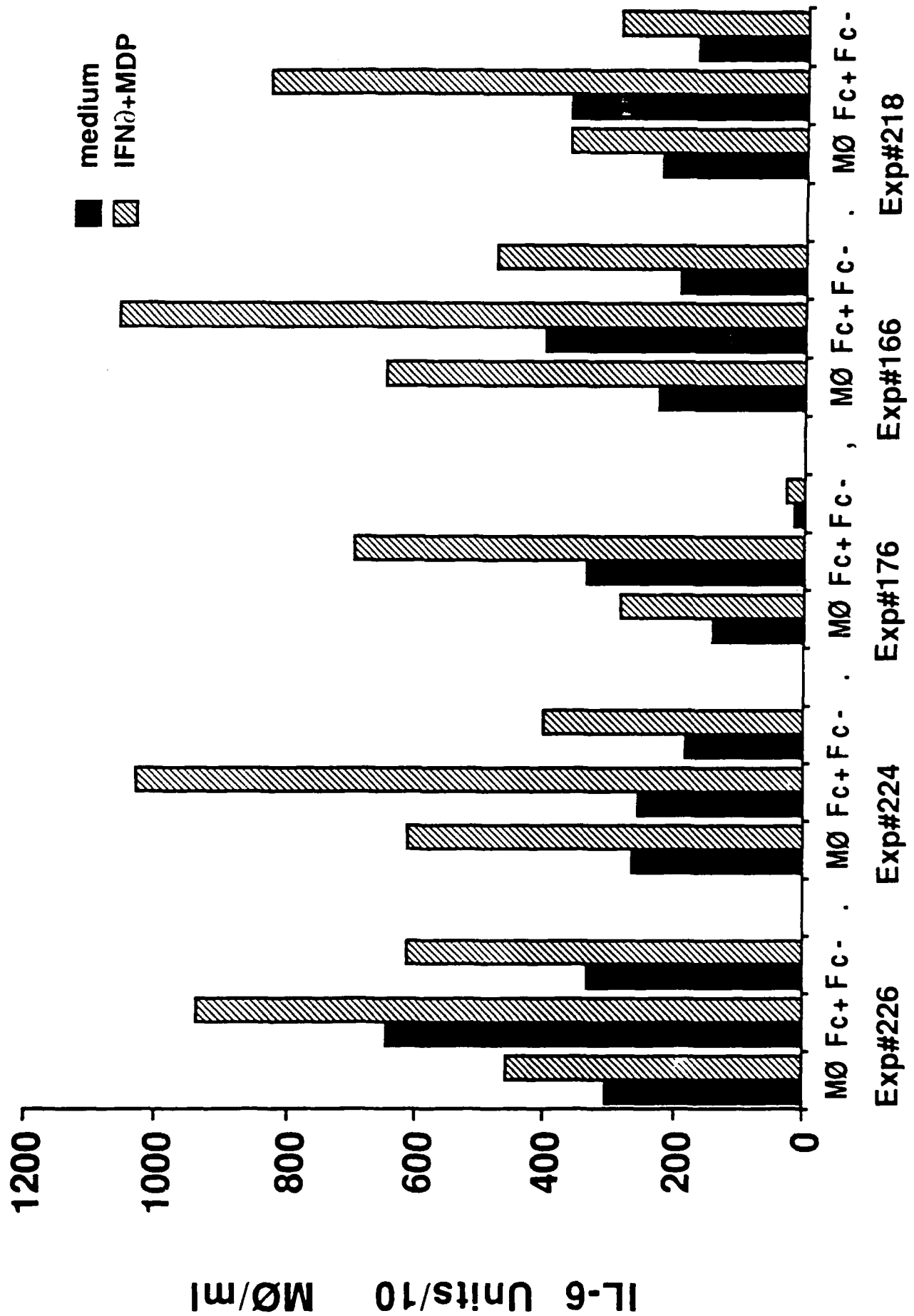
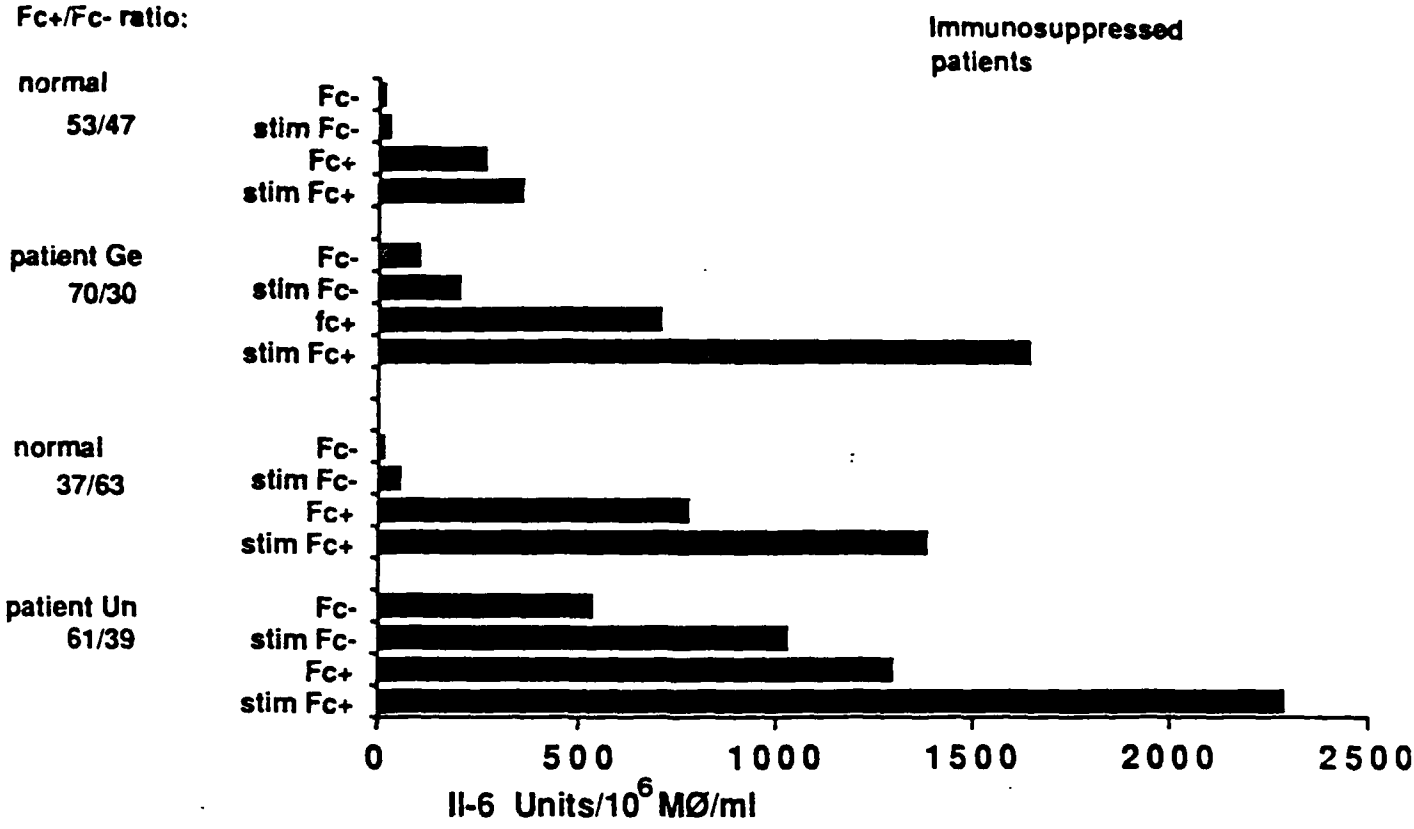

IL-6 Units/10 MØ/ml
1200
1000
800
600
400
200
0
medium
IFNϑ+MDP
MØ Fc+ Fc-
Exp#226
MØ Fc+ Fc-
Exp#224
MØ Fc+ Fc-
Exp#176
MØ Fc+ Fc-
Exp#166
MØ Fc+ Fc-
Exp#218

FIGURE 5


Fc+/Fc- ratio:
Immunosuppressed patients
normal 53/47
patient Ge 70/30
normal 37/63
patient Un 61/39
Fc-
stim Fc-
Fc+
stim Fc+
0
500
1000
1500
2000
2500
Il-6 Units/10^6 MØ/ml

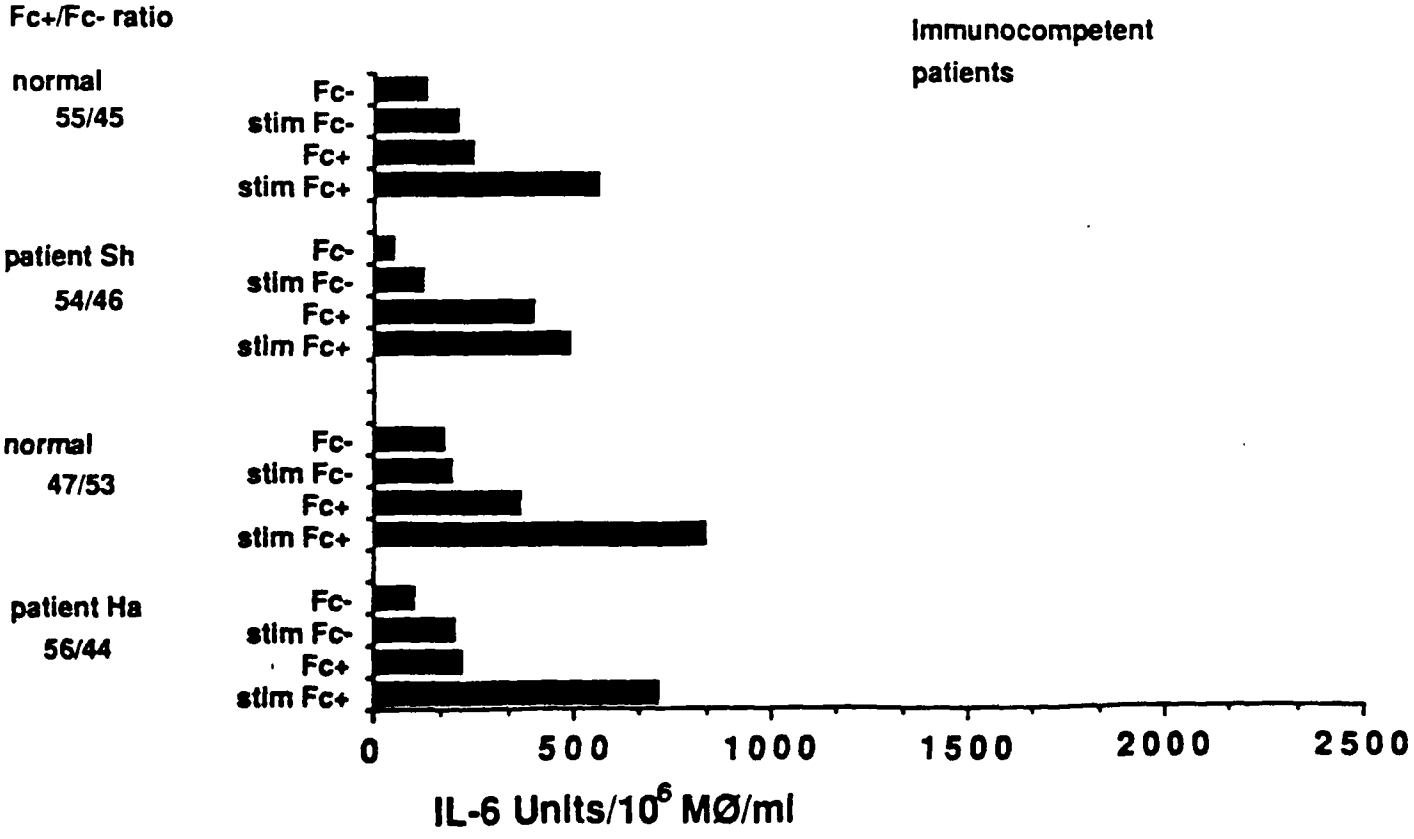
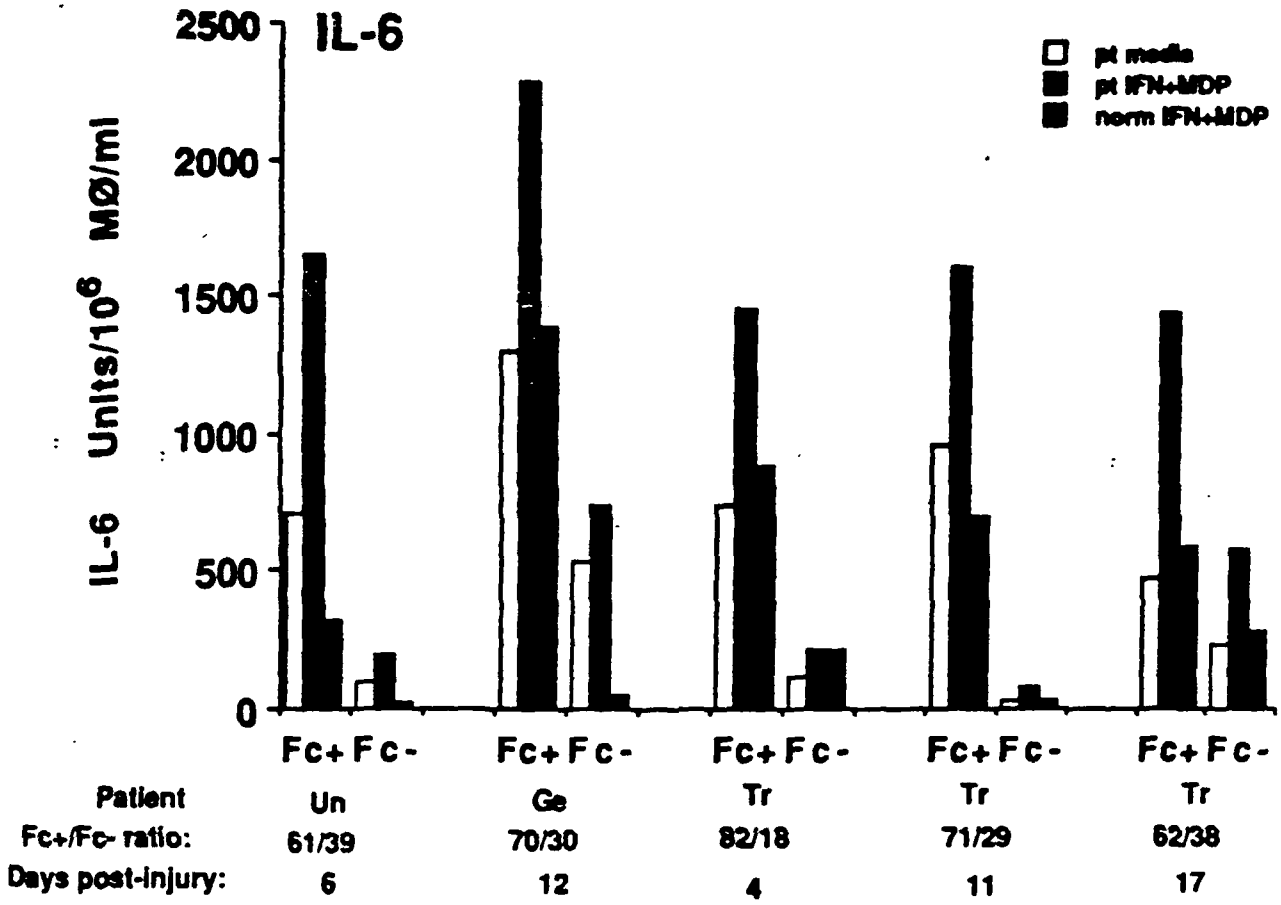

Fc+/Fc- ratio
Immunocompetent patients
normal 55/45
patient Sh 54/46
normal 47/53
patient Ha 56/44
Fc-
stim Fc-
Fc+
stim Fc+
0
500
1000
1500
2000
2500
IL-6 Units/10^6 MØ/ml

FIGURE 6

MØ IL-6 is unaffected by elevated MØ PGE_2 in trauma patients



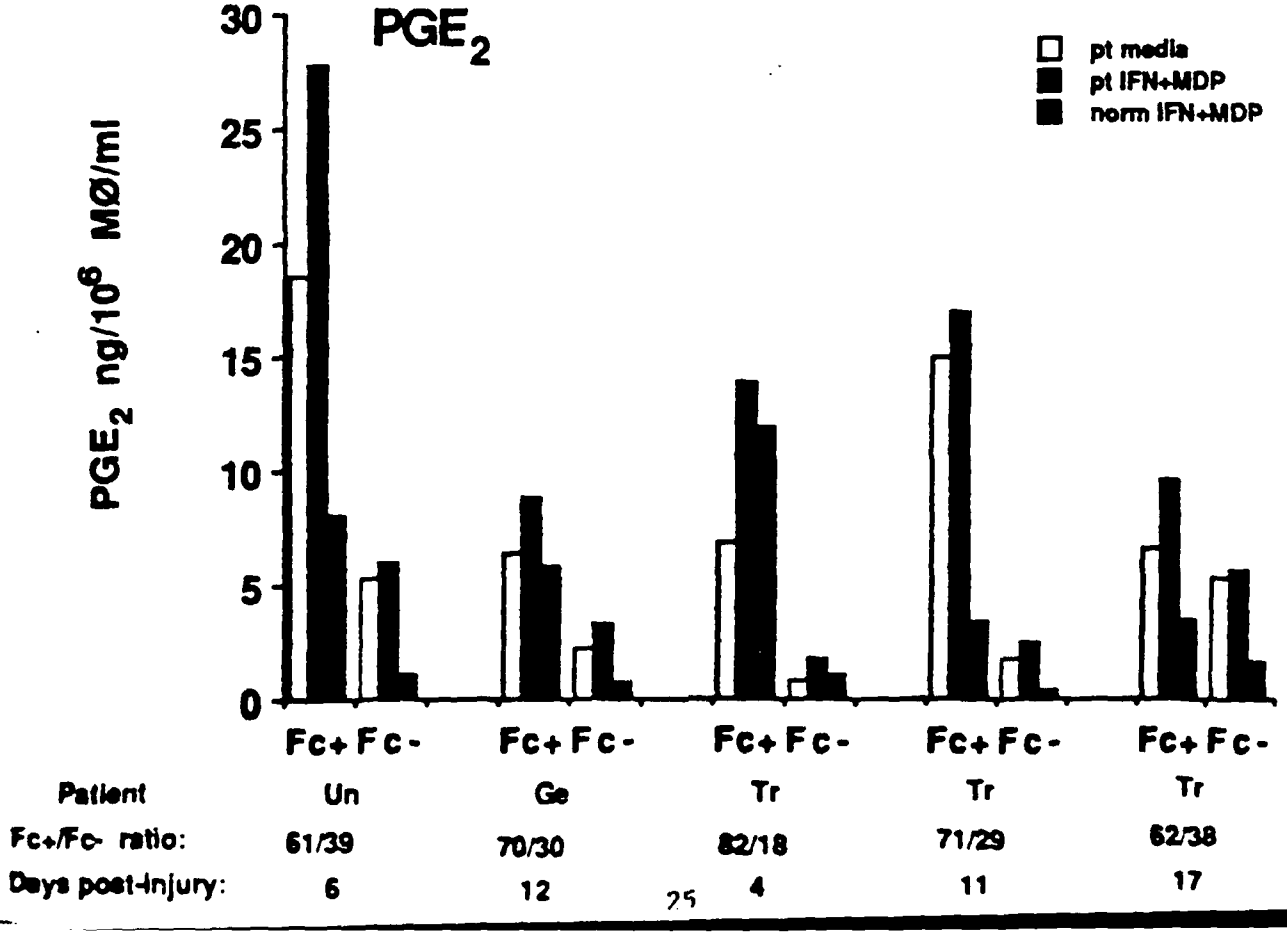


FIGURE 7

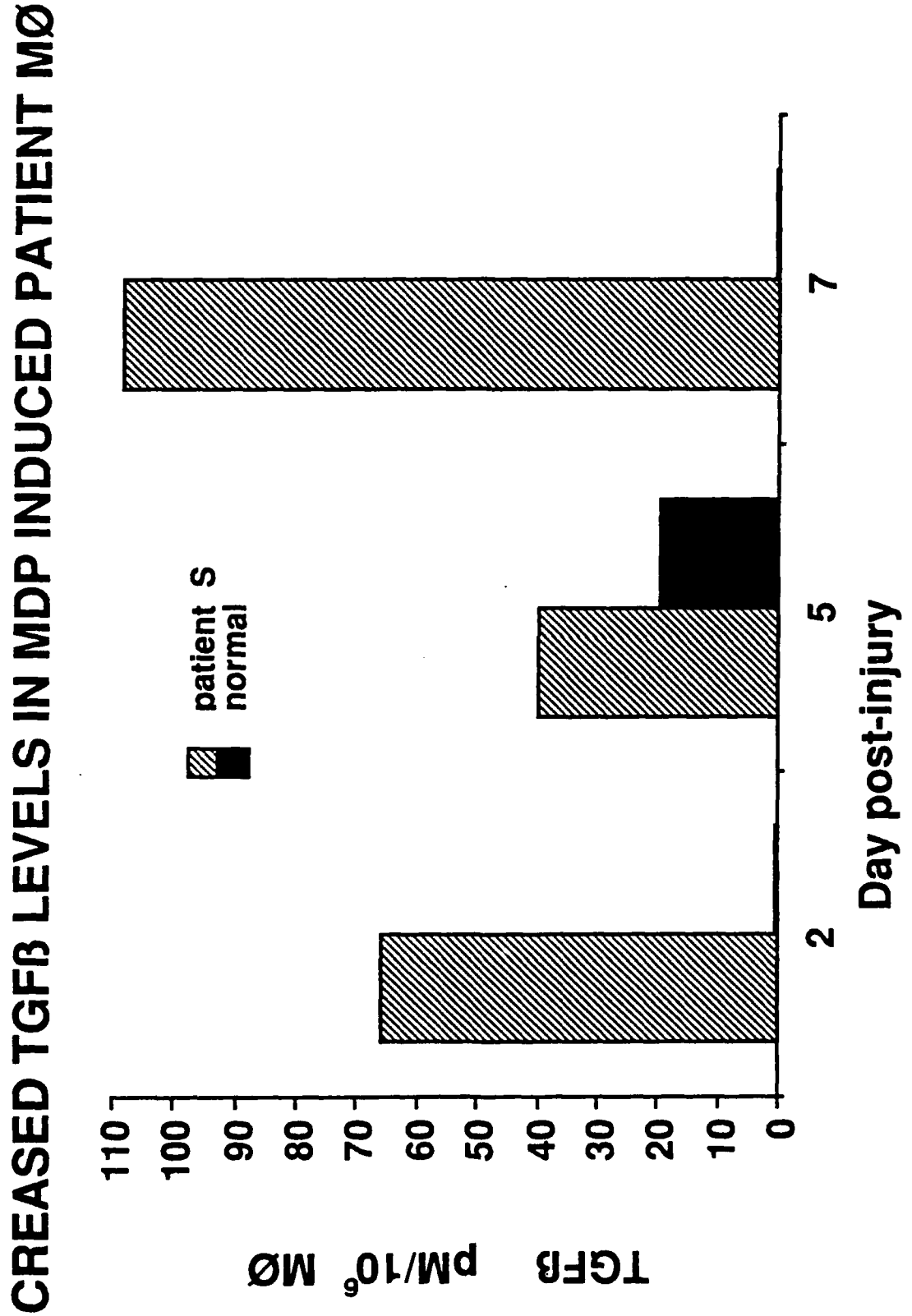
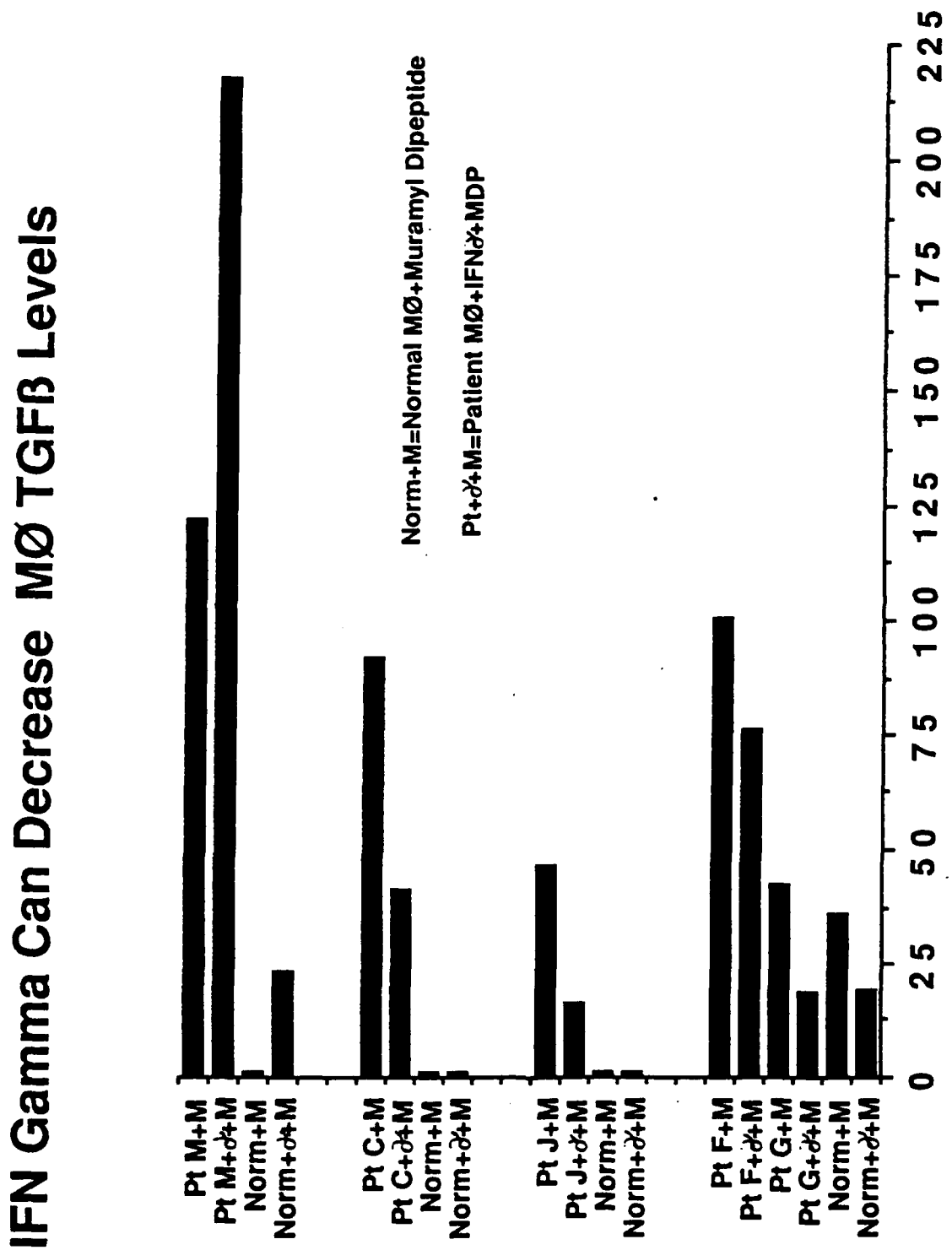

INCREASED TGFß LEVELS IN MDP INDUCED PATIENT MØ
TGFß pM/10^6 MØ
110
100
90
80
70
60
50
40
30
20
10
0
patient S
normal
2
5
7
Day post-injury

FIGURE 8


IFN Gamma Can Decrease MØ TGFß Levels
Norm+M=Normal MØ+Muramyl Dipeptide
Pt+∂+M=Patient MØ+IFN∂+MDP
Pt M+M
Pt M+∂+M
Norm+M
Norm+∂+M
Pt C+M
Pt C+∂+M
Norm+M
Norm+∂+M
Pt J+M
Pt J+∂+M
Norm+M
Norm+∂+M
Pt F+M
Pt F+∂+M
Pt G+M
Pt G+∂+M
Norm+M
Norm+∂+M
0
25
50
75
100
125
150
175
200
225
pM TGFß/10^6 MØ

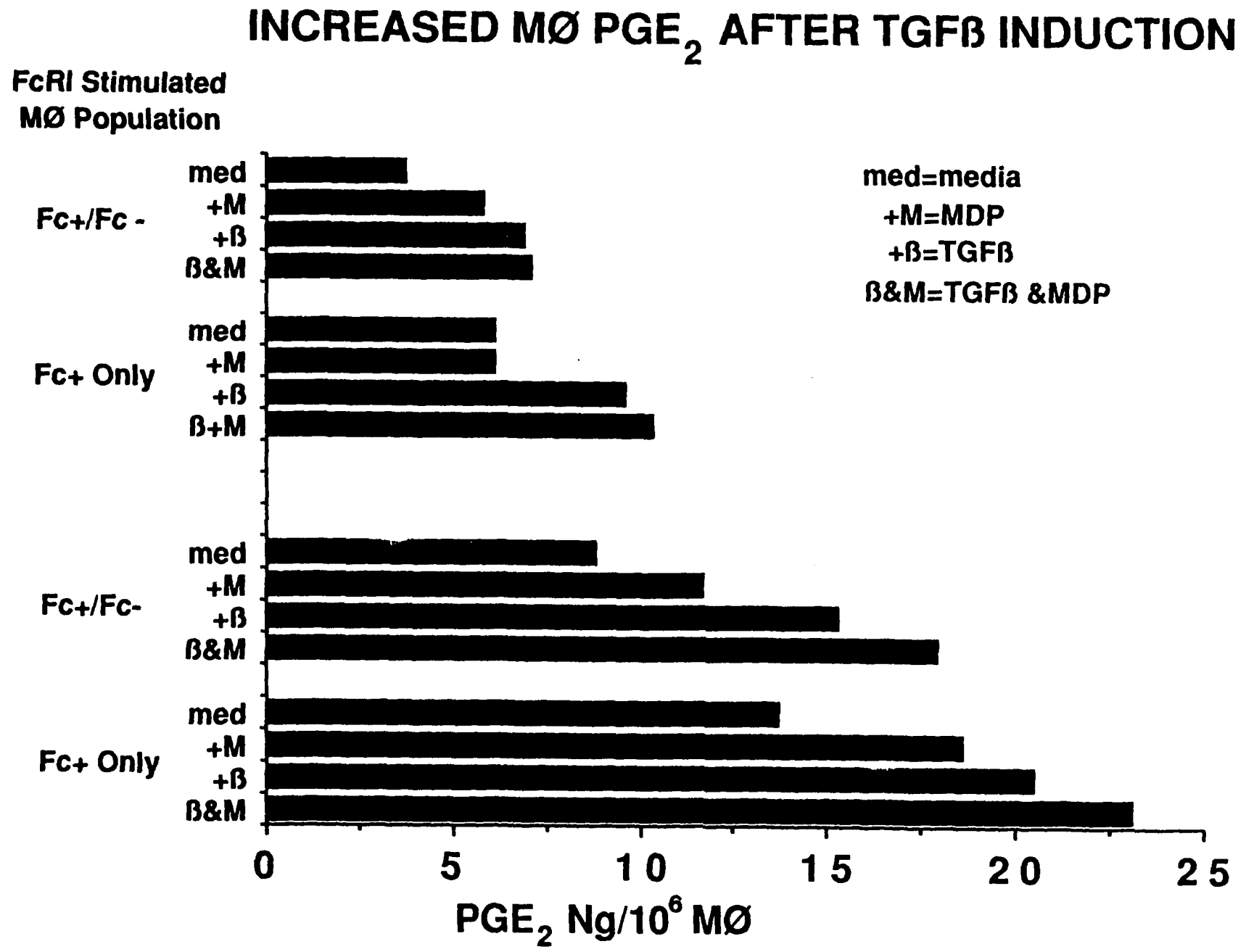

INCREASED MØ PGE2 AFTER TGFß INDUCTION
FcRI Stimulated
MØ Population
med=media
+M=MDP
+ß=TGFß
ß&M=TGFß &MDP
Fc+/Fc -
med
+M
+ß
ß&M
Fc+ Only
med
+M
+ß
ß+M
Fc+/Fc-
med
+M
+ß
ß&M
Fc+ Only
med
+M
+ß
ß&M
0
5
10
15
20
25
PGE2 Ng/10^6 MØ

FIGURE 6

TGFß Increases Separated MØ's Cell associated TNF Levels

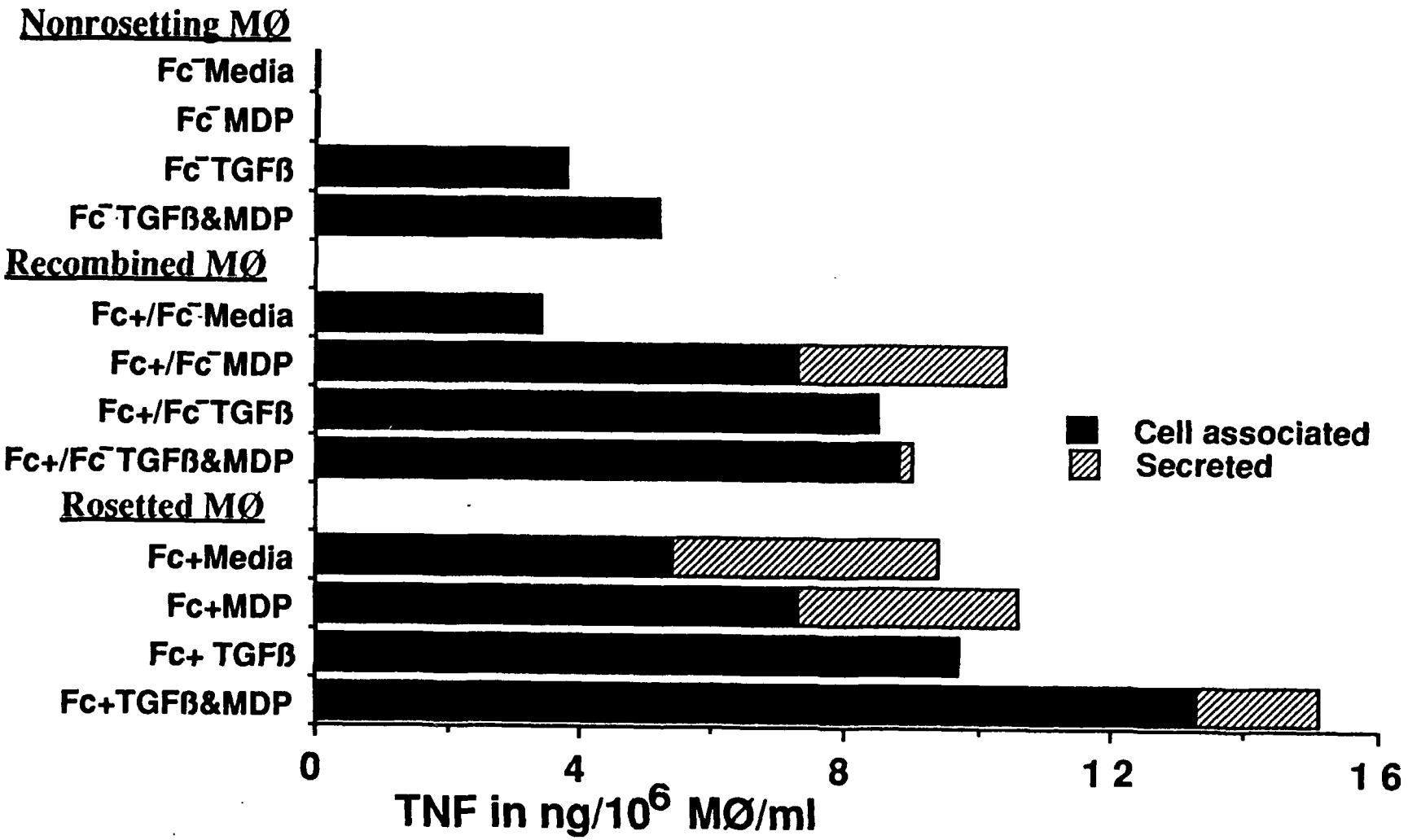
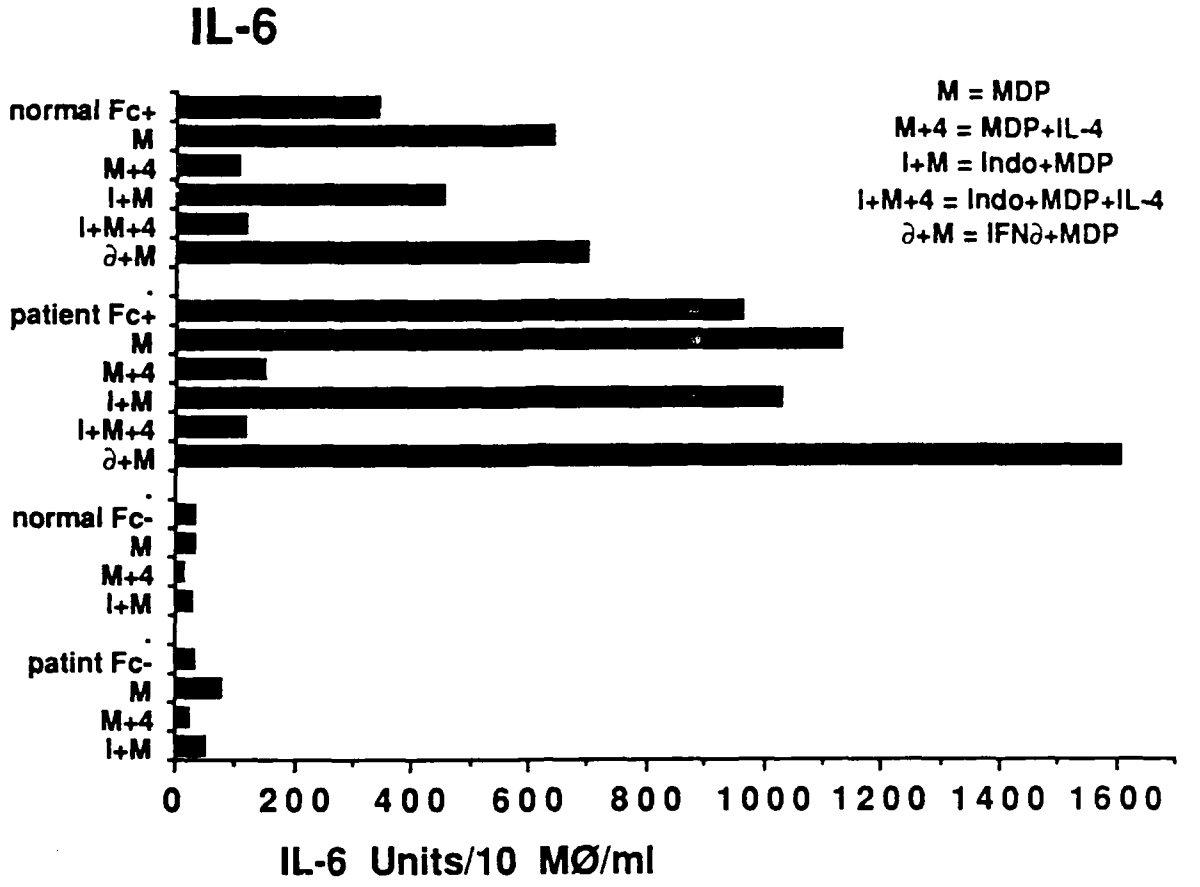
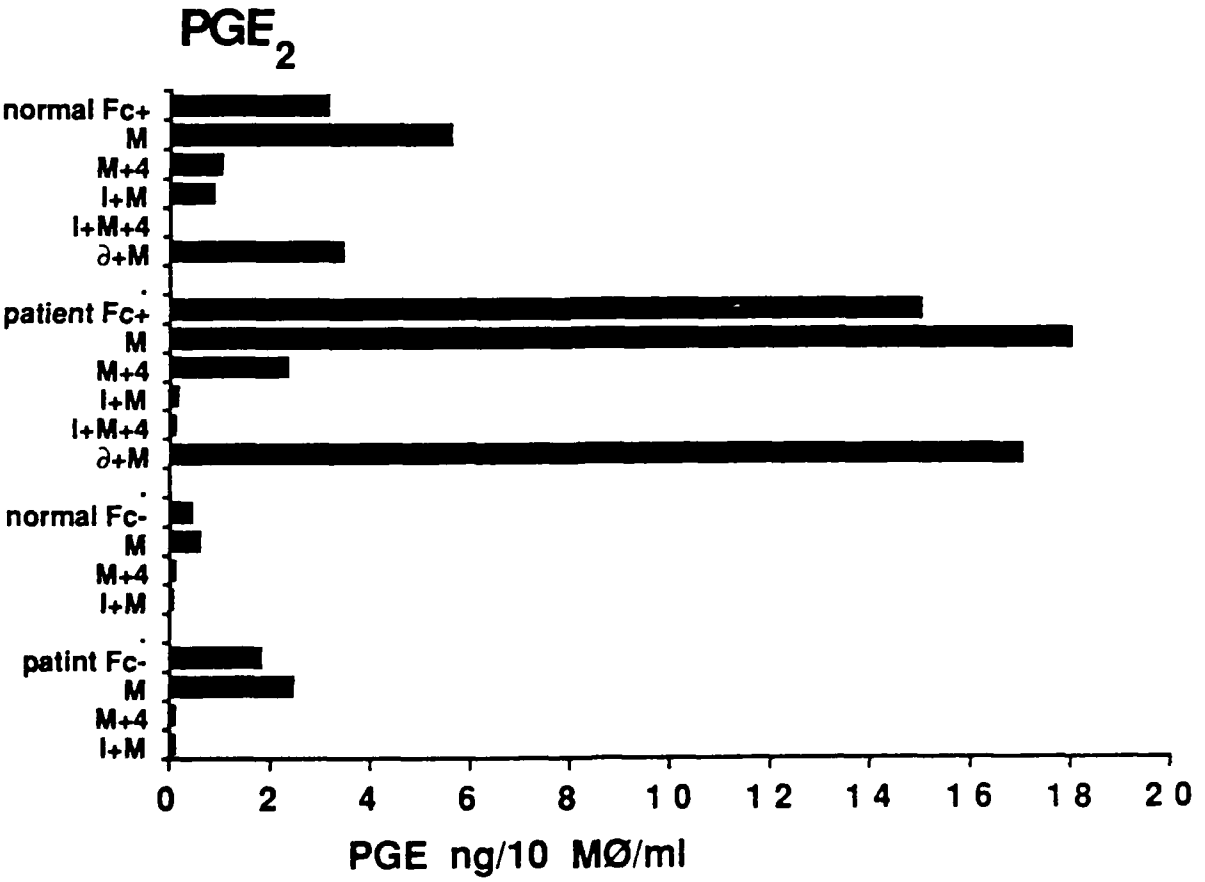


FIGURE 10

FIGURE 11


IL-6
M = MDP
M+4 = MDP+IL-4
I+M = Indo+MDP
I+M+4 = Indo+MDP+IL-4
∂+M = IFN∂+MDP
normal Fc+
M
M+4
I+M
I+M+4
∂+M
patient Fc+
M
M+4
I+M
I+M+4
∂+M
normal Fc-
M
M+4
I+M
patint Fc-
M
M+4
I+M
0 200 400 600 800 1000 1200 1400 1600
IL-6 Units/10 MØ/ml


PGE2
normal Fc+
M
M+4
I+M
I+M+4
∂+M
patient Fc+
M
M+4
I+M
I+M+4
∂+M
normal Fc-
M
M+4
I+M
patint Fc-
M
M+4
I+M
0 2 4 6 8 10 12 14 16 18 20
PGE ng/10 MØ/ml