

Summary and Conclusions



Summary and Conclusions

Genesis of autoimmune diseases is complex; in the amphitheatre of a responsive and responding substratum of immune system, the dramaturgy is constituted by artists, some of whom remember their lines well, some require a cue, some desire a cue while few others are endowed with a proclivity to forget their dialogue; among the latter are some with a transmuted dramatis personae, intrinsically chaotic and disseminating chaos ending up in premature curtains that may occur after a few scenes, many acts or at the very last scene. Agent provocateur to initiate the chaos include many, ranging in spectrum, genetic susceptibility to social status to environmental factors. The list of environmental factors that trigger autoimmune diseases in genetically susceptible individuals has grown in the recent years and is far from complete. The possible intervention of the environment in the triggering of these diseases is ever more perceived by clinicians. In this study, the effect of certain environmental factors/microbial antigens on proportions of different T-lymphocyte subsets and cytokine secretion by PBMCs of pemphigus, systemic sclerosis and systemic lupus erythematosus patients, before and after specific immunosuppressive therapy, were studied *in-vitro*. Also studied were twenty two single nucleotide polymorphisms in thirteen cytokine genes in these patients. The main findings of the study are:

I. Pemphigus:

- T-lymphocytes from pemphigus patients proliferated when stimulated with SPEA and SEB at varying concentrations. The proliferation was observed only in the CD3⁺CD4⁺ population of T lymphocytes. Also, a significant increase was observed in the memory (CD45RO⁺) phenotype of the CD4⁺ T cells in these patients on stimulation with SPEA and SEB. Further, the cytokine profile in response to bacterial sAg-stimulation seems to be dominated by the IL-2- and IFN- γ producing cells in pemphigus patients, while in healthy controls it is dominated by cells producing IL-4 and IL-10. Apparently host factors influence the quality and magnitude of cellular responses, and their cytokines, to these sAgs. Consequently infection-linked sAg-activity has a decisive bearing underpinning the clinical outcome of the infection which in turn suggests an important role for

sAg-activated T cells and cytokines in triggering episodes of clinically active autoimmunity in pemphigus patients. An attempt to contain sAg-producing bacterial colonizers may be worthwhile, and may decrease the severity by inhibiting triggering factors and prevent frequent relapses.

- Stimulation of PBMCs with CMV antigen resulted in increase in CD4⁺ population of T cells, particularly CD4⁺CD45RO⁺ subset, as compared to healthy controls. Stimulation with CMV antigen also lead to an increase in the production of IFN- γ . The high production of interferons and other cytokines in response to CMV antigen in pemphigus may hyper-activate the immune system and, through aberrant cell-mediated and humoral immune responses, lead to the antibody-mediated acantholytic disorder. As herpes virus infections are detected inconsistently in pemphigus patients, infection with CMV may be an occasional factor triggering the outbreak or exacerbation of the autoimmune disease in a non-specific way in genetically susceptible hosts.
- Increase in percentages of CD4⁺CD45RO⁺ T-lymphocytes in pemphigus patients upon stimulation with CA and PPD was observed. PPD stimulation also resulted in an increase in CD3⁺CD4⁺ T-lymphocytes; however response of these cells to CA stimulation was poor. A decreased Th1 and Th2 cytokine response upon stimulation with both the rAgs led us to propose that host variation in cytokine responses to the same antigens may explain the starkly different T cell responses in pemphigus patients and controls and also emphasize the continuing susceptibility of these patients to co-infections with *Candida*. Once exposed, the overall T-cell clonal size appears to remain stable. This information will help us better understand the pathogenesis of pemphigus and may be helpful for optimization of therapy in these diseases.
- Higher levels of OCPs like β -HCH (isoform of hexachlorohexane), α -endosulfan (a form of endosulfan) and p,p'-DDE (a metabolite of DDT) were observed in the blood of pemphigus patients as compared to healthy controls. HCH and DDT exposure caused specific reduction in CD8⁺CD45RA⁺ and CD4⁺CD25⁺ T-lymphocyte subpopulations in pemphigus patients. The other prominent finding was a strong reduction in Th1 (IL-2 and IFN- γ) cytokines upon exposure to these

OCPs *in-vitro*. These findings indicate that HCH and DDT have a significant impact on Th1 lymphocytes. Impaired production of these cytokines might favor infections and production of autoantibodies. We therefore speculate that the systemic absorption of the pesticide after the topical contact may also be one of the factors triggering the immunological mechanism in pemphigus.

- The proportion of CD4⁺CD25⁺ regulatory T cells (Tregs) in all pemphigus patients was severely reduced. These observations were further confirmed by diminished protein expression of Foxp3 (forkhead box P3; a specific marker of natural T regulatory cells) in the CD4⁺CD25⁺ T cell population in these patients.
- Immunosuppressive therapy caused a significant reduction in CD4⁺ T cells (both naïve and memory) and in CD8⁺ (naïve) T-lymphocytes upon stimulation with sAgs, rAgs and OCPs. An increase in CD4⁺CD25⁺ T-lymphocytes in response to sAgs and OCPs was also noted. Further, a significant reduction in IL-2 secretion after therapy was observed. This depletion of environmental antigen activated T cells induced by dexamethasone cyclophosphamide pulse (DCP)/dexamethasone pulse (DP) therapy can be associated in pemphigus with T-cell subset alterations, changes in the activation state, and induction of cells with regulatory properties. These results strongly suggest a differential effect of DCP/DP therapy on autoreactive versus pathogen-specific T cells. It cannot be excluded that the use of immunosuppressive treatments has a synergistic effect on humoral and cellular immune responses, including autoreactive CD4⁺ T cells, in treated patients.
- In this case-control study, significant differences in allele and genotype distribution were observed in IL1R pst1 1970 C/T, IL4Rα +1902 G/A, IFNγ +874 T/A, and TGF-β1 codon 10 T/C and codon 25 G/C gene polymorphisms. IL-1R1, IL-4Rα, IFN-γ and TGFβ1 SNPs show significant association with the disease.

II. Scleroderma (Systemic sclerosis):

- The potential role of bacterial sAgs in triggering T-cell activation was confirmed/supported by a higher expression of CD4⁺ T-cells in patients with SSc. High expression of activation markers CD45RA (naïve) and CD45RO (memory) on CD4⁺ T-cells of SSc patients, as compared to controls, suggests that both these

cell populations might be involved in autoimmune pathogenesis of SSc, and that effector T-cells involved in SSc presumably exist in the CD4⁺ T-cell subpopulation. IL-4 secretion showed a significant increase in SSc patient PBMCs with both the bacterial sAgs, while IL-10 increased only with SEB stimulation. In case of IL-2 and IFN- γ , considerable increases were observed in SSc patient PBMCs with both these sAgs. The differences in these cytokine levels in patient PBMCs as compared to controls are probably due to the heterogeneity within SSc. Measuring these cytokine levels in patients would be helpful in predicting the development of detrimental clinical manifestations. The accumulation of sAg-reactive T-cells may result in an increased chance of relapses/resistance to therapy in these patients and, therefore, steroid/immunosuppressive therapy given to these patients should be carefully monitored.

- Upon CMV stimulation, the percentage of CD4⁺ T cells in SSc patients increased significantly, whereas the percentage of CD8⁺ T cells decreased. Further analysis showed higher expression of CD45RO on both CD4⁺ and CD8⁺ T cells. We also observed increase in percentages of CD4⁺CD25⁺ T cell population in these patients. Expression of IFN- γ and IL-4 was elevated in PBMCs with CMV antigen stimulation. Altogether, these findings suggest that the CMV driven cascade of events may be one of the factors leading to typical SSc T cell alterations and up regulation of fibrogenic cytokines. CMV could thus be a triggering factor of autoimmunity in genetically predisposed and infected SSc patients.
- CD4⁺ T-cells of SSc patients responded differently to common rAgs (CA and PPD) challenge *in-vitro*. Their proliferative response was not as high as was observed with bacterial sAgs challenge. Also, CD8⁺ T-cells and activation markers of SSc responded weakly to these antigens, as compared to controls. The poor response of CD45RO⁺ to rAgs perhaps reflects the dearth of functional memory cells which leads to ‘anergy’ in these patients. Anergy was observed in 16 of 20 SSc patients by skin delayed hypersensitivity test. There was a significant increase in IL-4 levels in SSc PBMCs upon rAg stimulation. IL-10 secretion on the other hand showed a considerable decrease in SSc PBMCs. Such a paradoxical suppression of peripheral immune responses, along with a high degree of anergy,

may contribute to susceptibility to and persistence of Th2 responsiveness in the SSc disease through sAg/environmental antigen challenge. The functional recovery of CD8/memory pool of T-cells and the cytokine response after specific therapy may indicate a reversal of the chronicity of the disease.

- HCH (α -, β - and γ) and p,p'-DDE were found in higher levels in the blood of SSc patients than healthy controls. *In-vitro* treatment with either HCH or DDT caused a significant increase only in levels of CD8⁺CD45RO⁺ T-lymphocytes as compared to healthy controls. Neither HCH nor DDT exposure was able to alter significantly the secretion of IL-2, IL-10 or IFN- γ in SSc PBMCs but an elevation in IL-4 secretion was conspicuous. Both the OCPs were able to reduce the levels of all these cytokines in healthy control PBMCs. SSc has often been considered a Th2 cytokine disease. Th1 cytokines such as IFN- γ have anti-fibrotic effects, while Th2 cytokines such as IL-4 have pro-fibrotic effects. These results collectively show an association between HCH and DDT levels in the blood and decreased lymphoproliferative activity. Thus, SSc patients are less able to initiate or augment an immune response to foreign antigens. When there is substantial suppression of T and B cell function, there is increased susceptibility to infection. The present study also confirms that persistent exposure to these pesticides can affect cytokine production and thereby increased disease susceptibility.
- Although we observed higher percentages of CD4⁺CD25⁺ T cells in SSc patients than healthy controls, the expression of Foxp3 was significantly reduced in them. Foxp3 is quantitatively related to a reduction in functional suppression induced with sub-optimal T cell receptor ligation.
- Immunosuppressive therapy decreased the CD4:CD8 T cell ratio in SSc patient PBMCs. This was also apparent after sAgs stimulation of SSc patient PBMCs. A predominance of CD4⁺CD45RO⁺ T cells and a deficiency of naïve CD4⁺CD45RA⁺ T cells in these patients after therapy, in unstimulated as well as in sAg, rAg and OCP challenged PBMCs, was highlighted in our study. An elevation in IFN- γ levels in unstimulated SSc PBMCs was conspicuous with a corresponding decrease in IL-10, and no change in IL-4, after immunosuppressive therapy. This increase in the levels of IFN- γ was also demonstrated when patient

PBMCs were stimulated with sAgs, rAgs and PHAM after therapy. Reduction of CD4:CD8 T cell ratio in PBMCs *in-vitro*, in conjunction with augmentation of CD4⁺CD45RO⁺ T cells after immunosuppressive therapy, may indicate diminished autoimmune response and activated pathogen-specific response in these SSc patients. These results also show a decrease of profibrotic and proinflammatory cytokines (IL-2) in SSc patient during immunosuppressive treatment.

- The study showed a significant correlation of single nucleotide polymorphism in IL-1 β -511 and +3962, and TNF α -308 and -238 with systemic sclerosis.

III. Systemic lupus erythematosus:

- Using SPEA and SEB as a prototype sAgs, we have demonstrated in this study that the interplay among PBMCs and sAgs profoundly affects the magnitude of sAg-driven polyclonal T cell responses and cytokine secretion *in-vitro*. Our results indicate that sAg-activated CD4⁺ T cells may be playing a role in immune dysregulation thereby contributing to autoimmune phenomenon in SLE. A high CD45RA or CD45RO expression on CD4⁺ and CD8⁺ T cells in SLE PBMCs may be related to perturbed homeostasis of these T cells in SLE patients. Changes in T-cell phenotypes, including the CD4/CD8 ratio and CD45RA and CD45RO expression, may be good indicators of therapeutic efficacy in SLE patients. Our results also suggest that decrease in the frequency of CD4⁺CD25⁺ (Tregs) might play a specific pathogenic role in SLE. The increase in IL-2 and IFN- γ production in patient PBMCs with sAgs stimulation are presumably due to potency of these antigens in polyclonal activation of large numbers of cells, possibly including autoreactive T cells. Augmented IL-4 production was more conspicuous in SLE PBMCs than control cells upon sAgs stimulation; inversely IL-10 registered high increase in control PBMCs than SLE cells. This sAg-promoted IL-10-production in healthy PBMCs exert an immunoregulatory effect by suppressing IFN- γ production. However, such a regulation of cytokine response to sAg stimulation has gone astray in SLE PBMCs.

- Stimulation of SLE PBMCs with CMV antigen caused high proliferation of CD4⁺CD45RO⁺ among CD4⁺ and CD8⁺CD45RO⁺ among CD8⁺ T cells. The CD4:CD8 ratio was also significantly higher as compared to control PBMCs. The overproduction of IFN- γ coincides with disease flare in SLE patients, but has a protective effect. IFN- γ mediated IL-2 release through its T cell growth factor activity ensue release of proinflammatory cytokines resulting in autoimmunity.
- Our data suggests that a major factor in the differential response to rAgs of the two populations of PBMCs may be a lack of cells capable of recognizing the antigen in SLE patients. These results clearly indicate that in healthy controls CD45RA⁺/CD45RO⁺ balance is tilted towards the more stable resting state (i.e., the CD45RA⁺ pool) to prevent eventual loss of response to subsequent antigen challenge. Alternatively, in SLE patients, it seems likely that the eventual fate of primed CD45RO⁺ T cells *in-vivo* is overwhelmingly death by apoptosis. Increased production of IL-2 and IFN- γ in SLE PBMCs upon stimulation with CA and PPD indicates that quantitative differences in proportions of CD4⁺ and CD8⁺ T-cell subsets and their interaction may be leading to functional differences in immune response through production of different cytokines. Increased IL-10 levels in unstimulated PBMCs of patients with SLE when compared with control cells is possibly attributable to an increase in IL-10 production by CD4⁺CD45RO⁺ memory T cells. Both CA and PPD decreased IL-10 production in SLE PBMCs. The depletion of T regulatory cells in SLE patients may be one of the causes for IL-10 diminution in their PBMCs *in-vitro*. In spite of this complexity, our data should be useful as a basis for defining the change in cytokine expression in PBMCs responding to commonly used stimuli in normal and SLE states.
- OCPs—specifically, HCH and DDT—were found in high levels in blood of SLE patients. It is likely that higher levels and prolonged exposure to these pesticides may have significantly influenced T-lymphocyte sub-sets and cytokine expression *in-vivo*. The high IL-10 expression in these patients was independent of disease severity and could affect production of IL-2 or IFN- γ in these hosts. Furthermore, incessant low dose exposure to these OCP may functionally handicap the patients' cells to respond to extraneous cytokines, resulting in induction of a form of cell

exhaustion or anergy. Although the clinical significance of these findings is uncertain, the described changes in lymphocyte sub-sets and cytokine formation by these cells could be critical in the development/exacerbation of SLE or other autoimmune diseases.

- We observed that patients with SLE had decreased CD4⁺CD25⁺ T cells. This coincided with decreased expression of Foxp3. These findings indicate that autoimmunity in SLE patients is not necessarily due to a deficiency of Treg cells per se, but rather, is due to their apparently impaired suppressive function in the absence of Foxp3 and in the presence of IFN- γ -producing antigen presenting cells.
- While no significant changes were observed in CD4⁺ T cells in unstimulated PBMCs of SLE patients after immunosuppressive therapy, this was not the case with respect to CD8⁺ suppressor/cytotoxic T-cell subsets. With control of the disease, CD8⁺ T cells increased, but the values were still reduced than the healthy controls. This shows the relation of these CD4⁺ T-cell subset abnormalities to multiple immune aberrations in SLE patients. Therefore, it is possible that an elevated expression of CD45RO on CD4⁺ T-cell subsets may precede other immunological abnormalities in SLE and may be of pathogenetic significance. However, the mechanism for their induction remains unknown; CD4⁺ helper/inducer T cells are required for delayed hypersensitivity reactions, IL-2 production, immunoglobulin synthesis, and activation of CD8⁺ suppressor/cytotoxic T cells needed for homeostatic regulation of antibody production. In this context, immunosuppressive property and the ability of the drugs to influence specifically, CD4⁺ T-cell subsets and the immune system may play a central role in controlling this disorder in humans. Clinical improvement in SLE has been associated with the reduction in productions of IFN- γ and slightly increased IL-4 production. Immunosuppressive therapy given to the patients in our study caused a significant decline in secretion of all the cytokines analyzed except IL-4, where no significant change was observed. The results obtained in this study indicate that measurement of IL-4 and IL-10 levels in circulation of SLE patients after immunosuppressive therapy may be a potential tool for monitoring clinical progress.

- Cytokine genotyping analysis showed weak association of TGF β 1 and IL-10 polymorphisms with the SLE.

We propose that immune regulation is a multi-layered complex interaction with checks and counter-checks with inextricably linked functional and quantitative effects on responding immune effector cells. Each layer of regulatory interactions is differently susceptible to nocuous stimuli. Extent of susceptibility of one layer determines the functional optima of the activity it regulates. Some of the affectations are circumscribed and regional, others are global and yet others have a domino effect affecting many arms of immune reactivity in a cascade. A combination of all these in a background of genetic susceptibility and environmental exposure to nocuous triggers, punctuated by intensity and intermittency of such exposures unfolds as clinical disease with inevitable variations linked to undercurrent diversity in immune reactivity of responder cells.