First of all, I would like to thank the organisers for inviting me and especially doctor Barth who seduced me becoming interested in the disease. I am still feeling rather ignorant, being involved in the study of Aicardi-Goutières Syndrome for only six months. The findings have made me curious and I will tell you a bit about what we have done until now.

Many of the previous speakers have already mentioned how the syndrome of Aicardi-Goutières is defined and which criteria make a diagnosis most plausible. As an immunologist, I was most interested by the chronic CSF lymphocytosis and the question posed to me was what this phenomenon could represent? Knowing that it was non-infectious in nature – at least that was the information at that time – and that there was an increased level of interferon alpha that could be measured, especially in the early phase of the disease, both in serum and in the spinal fluid. What could that mean and was there a relation between the increased interferon alpha and the lymphocytosis or the clinical manifestations?

First of all, what does interferon alpha mean for immunity? To answer this question I should explain to you some basic concepts and understandings about interferons and –in addition– give some overview of the immune system and the players of our interactive defense system.

As has already been alluded to, there are two types of interferons, type 1 and 2 interferons, and of course I will mostly deal with the role of interferon alpha in human immunity. As already mentioned, the interferon alpha and the single interferon beta gene are located on the short arm of human chromosome 9, i.e. located on 9p21. About the transcriptional regulation of these interferon alpha genes, something has already been mentioned. Most single- and double-stranded RNA as well as the DNA viruses are able to induce interferon alpha and beta production in whatever cell they infect. It is not so that the interferon alpha is a typical leukocyte interferon but it can be produced by probably most virus-infected or stressed tissue cells.
The induction of type 1 interferons is very rapid. Within hours, there is maximal or optimal production of interferon alpha and its induction is transient in nature. What is especially important is that it does not necessarily require protein synthesis for its induction, because all the transcription factors needed for interferon alpha synthesis are already available and pre-exist in the cytoplasm or the nuclei. Transcription of the interferon alpha and beta genes is regulated by the binding of a complex of transcription factors and transactivating factors that bind to and modulate the promoter region of the interferon gene. The composition of the binding initiation complex for gene transcription defines to a large extent the efficiency and quality of the process.

For instance, the human viral response element (VRE) for the interferon alpha gene has some docking site for interferon regulatory factors (IRFs) and these are known to be the positive regulatory domains (fig. 1A). We are at the beginning of understanding what factors are predominantly involved in transcription of interferon alpha or interferon beta. And a better understanding hereof is important in the light of the question: is there a dysregulation of interferon alpha production responsible for Aicardi-Goutieres syndrome?

![Fig. 1. A: Human chromosome 9: interferon-alpha Type I. B: Human chromosome 9: interferon-beta. VRE: virus response element. IRF: interferon regulatory factor. PRD: Positive regulatory domain.](image)

The same intricate and complex transcription machinery is progressively being identified in case of interferon beta (fig.1B). As can be expected and by analogy to interferon beta, more transcription factors for the interferon alpha gene will be discovered in the near future and might play a role in this disease.

Of course, the type 1 interferons are known to have antiviral functions and there are several factors being induced by interferon alpha, protecting a cell system or a tissue from viral replication and spread and these viruses as has been alluded to by doctor Di Andrea.

The interferon alpha isoforms, of which there are at least some 20, and interferon beta, of which only one isoform exists, signal through a similar receptor complex and this receptor complex is consisting of a R1 and R2 subunit. One is the binding receptor subunit and the other is required for transmitting the signals into the cell. Both are
linked to protein tyrosine kinases (PTK), both homologous and hence members of the Janus family of PTK.

And these, after causing the two subunits to dimerise, they ultimately result in phosphorylation of the so-called signal transducer and they activate transcription proteins, STAT proteins. What does this mean for immunity?

When focussing on lymphocytes, binding of interferon alpha to its receptor will lead to signaling and cellular activation.

In case of T cells, we can distinguish several subpopulations of T cells with different and specific functions within the immune system. Both pro-inflammatory as well as helper functions are ascribed to specialized sets of CD4+ T cells, being either involved in inflammatory reactions (e.g. a positive Mantoux test after former contact with mycobacterial antigens) or in the generation of antibodies by B cells (e.g. after vaccination or infection). These specialized cell types produce a set of cytokines involved in the functional activity of the cells by which these cells are recognized and designated an inflammatory T helper 1 or Ab-steering or allergy-related T helper 2 cell type, respectively. In fact, a further subdivision of the CD4+ T cells into subsets of non-polarized T cells, regulatory T cells, and follicular T cells involved in true germinal center B cell help, but these cell types are beyond the scope of this presentation. Finally, the cytotoxic effector function against virus-infected cells is mostly but not exclusively ascribed to the CD8+ T cell fraction.

These functional T cell programmes are, however, not yet present in every T cell but ought to be induced before becoming irreversibly imprinted, by the first steps of T cell activation. The outcome as to which programme and cytokine signature a cell will eventually get, is heavily dependent on the antigenic stimulus per se, the amount of antigen, the way by which the antigen is presented, and the environment in which this first encounter with the antigenic stimulus occurs. In this developmental T cell process, interferon alpha will activate and skew naive CD4+ T lymphocytes into so-called T-helper 1-like cells, able to produce and release cytokines causing local and -when abundant- systemic inflammation. This type of CD4+ T-helper 1 cells is usually responsible for the clearance of intracellular pathogens and inflammatory hypersensitivity reactions (e.g. the aforementioned Mantoux skin test) as a reflection of their activity, but –if for instance recognising a self-antigen– also the neural tissue damage in case of multiple sclerosis. And there are more organ-based autoimmune diseases that are provoked by the action of so-called CD4+ T-helper 1 lymphocytes.

It is important to be aware of the following. The effect of interferon alpha on the human system is clearly different from the murine system and that is very important if you will
consider a murine test system for Aicardi-Goutieres syndrome. Such has been claimed for the interferon-alpha transgene model with specific expression restricted to the central nervous system. Mouse is, however, very different from humans with respect to interferon alpha responsiveness, as has been most explicitly shown for immune cells.

The interferon-alpha receptor consists of two subunits. They are bound to particular tyrosine kinases (JAK1 and Tyk2). Upon ligand binding to the receptor, these subunits allow the cytoplasmatic tyrosine kinases to cross-activate each other and various tyrosine residues of one the cytoplasmic tail of the R1-subunit of the interferon-alpha receptor. This sudden change in the cytoplasmic tails creates a docking site for the cytoplasmic Signal-Transduction-and-Activator-of-Transcription (STAT) protein STAT2, to be precise. There are seven STAT proteins, but this is the one that in particular binds easily to the interferon-alpha receptor through dock to one (Y466) of the various phosphorylated tyrosine residues of the R1 subunit.

After docking, the STAT2 protein also becomes phosphorylated through JAK1 kinase. In this way, there is an altered configuration by which STAT2 may interact with another cytoplasmic protein, STAT4. Binding of STAT4 to STAT2 is only possible in humans, so it does not exist in the murine system and will impact studies on interferon alpha in the mouse, since humans will have a different outcome. Once this binding of STAT4 to STAT2 has taken place, the phosphorylated STAT-dimer, a heterodimer, can move to the nucleus where it serves for transcriptional activation and induces, in case of lymphocytes, a cytokine response designated a T-helper 1 cytokine response (see later) (fig. 2B).

However, now we have only talked about STAT2 and STAT4 heterodimers, but there is more to it. STAT2 may also be bound and recognised secondarily by STAT1. A different heterodimer will be formed, leading to additional transcriptional activation causing other genes to be upregulated. Another STAT protein may dock to a different

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Fig. 2. JAK-STAT signalling. A: STAT heterodimerization; B: STAT translocation to the nucleus and induction of gene transcription, i.e. inflammatory cytokines in T cells.
site of the R1 subunit of the receptor causing the dimerisation of STAT3 to STAT2 or to itself forming homodimers, which—especially in tissue cells—induce a wide variety of gene regulatory and transcriptional activities.

The interferon alpha receptor signaling in itself is complicated, but I just wanted to emphasize that men and mouse are not identical in this respect. This is very essential if one considers an experimental animal model for the interpretation of the syndrome we are speaking of today, or the evaluation of drugs for its treatment. I have said that the interferon-alpha receptor complex induces transcription through the STAT complexes and, in human lymphocytes, the result is a different complex with different effects that we hardly have recognised and appreciated to date.

If one speaks about immunity and the type-1 interferons (alpha or beta), we can conclude that interferon alpha is helpful in the treatment of hepatitis. Although not always effective when used as a single drug, how does it work and are there side-effects known to its use? In-vivo use of interferon alpha results in the activation of the so-called innate immune system. The innate immune system is a primitive defense system that is not dependent on the strict rules of specific antigen recognition and the induction of a programme of differentiation from a naive to effector cell as mentioned for T cells. Yet, innate immunity contributes to the detection and elimination of for instance virus-infected and damaged cells, or malignant cells if low in HLA-antigens and exposed in the right fashion. These responses of the innate immune system are mainly due to the activity of natural killer (NK) cells. There are several factors that may activate these NK cells to produce cytokines that cause inflammation or at least an environment that makes virus-infected or damaged cells susceptible for further immune attack and definite elimination.

A set of surface markers on the cell can be used to detect whether the right number of NK cells are present in the blood (or tissues) and also to isolate or enrich for these NK cells and test their function. These markers are called the CD16 and CD56 antigens. Of course, NK cells have a myriad of different proteins on their surface. Once these cells have found their right target cell through surface molecules acting as receptors or adhesion molecules for intercellular contact, either a virus-infected and damaged cell, or a malignant and deregulated cell, may get killed. NK cells are subject to positive regulation by such surface molecules, of which CD16 is one, and negative regulation. In itself, the mechanism of control is little understood but an absolute requirement because in case of insufficient regulation, a host may end up with unnecessary tissue destruction or autoimmune disease. The NK cells can secrete a multitude of proteases, creating a proteolytic environment, which together with cytokines such as tumor necrosis factor
(TNF) and interferon gamma (IFN-γ), or death-inducing surface molecules such as Fas Ligand (CD95L), will finally kill the damaged cells.

As already mentioned before, interferon alpha also activates the adaptive immune system that will only recognise specific antigen determinants (in the context of the HLA-system). This CD4+ T helper cell can be induced in humans by the use of interferon alpha or beta as mentioned before, by the action of the STAT2-4 and STAT2-1 heterodimers.

Normally, naïve lymphocytes derive from the thymus, where these cells develop while passing the complex criteria of rigid positive-negative selection. This selection process acts to avoid self-reactive T cells from development and thus the risk of autoimmunity. In the same way, positively selected T cells of either the CD4+ helper or CD8+ cytotoxic quality are generated, enabling a host to react to a wide variety of different antigenic determinants. We are born with (almost) 100% naïve cells and during life we encounter all kind of antigens. After a first encounter and outgrowth, the immune system creates a system of primed responsiveness, known as immunological memory. When a host contracts the same or a similar virus, the immune system is more rapid and vigorous in its response and may kill or suppress the invading virus before it causes disease. Individuals normally suffer only once for instance from varicella or Epstein-Barr virus, because of a well functioning immune system and immunological memory to attack those virus-infected cells a second time preventing the virus from causing disease again.

T-helper 1 cells create a proinflammatory milieu in which the cytokine response is predominated by TNF, interleukin-12, interferon gamma (as a type-2 interferon), i.e. elimination through inflammation. Inflammation can be helpful but may also cause disease as can also occur in certain autoimmune diseases.

Last but not least, interferon alpha has also certain effects on the activation and killing potential of antigen-specific CD8+ cytotoxic T-cells. These cells use a similar machinery for virus-infected cells as the non-specific NK cells, but now as part of high-avidity recognition by specifically selected cells of the adaptive arm of the immune system. Although we are certain about the activating effect of interferon alpha on the effector functions of cytotoxic T cells, it is as yet unclear whether interferon alpha affects their differentiation from naive CD8+ T cells, as is the case for the naive CD4+ cell into inflammatory CD4+ T helper-1 cell.

T cells recognise a small peptide fragment of the antigen by the T-cell receptor. Both CD4 or CD8 function as a co-receptor for the recognition of the HLA-complex (also known as the molecules of the Major Histocompatibility Complex (MHC)) in which
this antigenic peptide is presented in the groove of the HLA molecule of either class II or class I, respectively. HLA class I molecules are present on virtually all cells with a nucleus; class II molecules are highly expressed on antigen-presenting cells such as the dendritic cells, that is present in all tissues at low number and samples the environment continuously bringing these antigen samples to the nearby lymphoid tissue (lymph nodes, mucosal lymphoid tissue or spleen) for a “screen” by the immune cells, such as macrophages as a tissue dwelling phagocyte engulfing damaged cells or invading pathogens, and activated B cells in the lymphoid tissue itself. Upon activation by proinflammatory cytokines, mostly interferon gamma, the tissue cells can also be induced to express class II HLA molecules to activate CD4+ T cells once primed by a virus-infection or otherwise.

Once the immune cells have been stimulated to proliferation and eventual differentiation into a cell with certain effector functions, these effector cells may use surface antigens (such as CD95L), cytokines, or perforin and proteases to exert a directly lytic activity on antigen-bearing tissue cells as in case of virus-infected cells. Thus, the activation and outgrowth of naive antigen-specific T cells in the secondary lymphoid organs will generate the correct signals for an immune cell to obtain a licence to kill in selective fashion, i.e. after migration into the tissues where the antigen is also present and recognised. Both CD95L and TNF may induce a pro-apoptotic signal in tissue cells, resulting in their elimination.

Once secreted from cytotoxic cells, perforin liberated from the secretory granules formed after differentiation of a naive immune cell into a competent effector T cell, forms small pores upon the specific recognition and binding of a target cell. These pores create an osmotic disequilibrium and also allow proteases to enter the target cell to cleave intracellular proteins into bits and pieces, unavoidably invoking cell death.
These are the effects of long-term use of interferon alpha in humans. Side effects consist mostly of fever, myalgia, and –rarely– anaphylactic reactions or neuritis. The latter is seen only in the very young and not undisputed.

What is the role then of interferon alpha in Aicardi-Goutieres syndrome; is there a role and how to inhibit type 1 interferons? Those were the questions while setting up a pilot study to test the use of monthly methylprednisolone (MP) infusions as a broad anti-inflammatory drug to block synthesis of interferon alpha – among many other cytokines. The rationale for using the MP is its potent anti-inflammatory activity and its wide use and experience in many autoinflammatory diseases. We treated 3 patients; only one had an increased level of interferon alpha as measured in serum and the cerebrospinal fluid by professor Lebon, at the start of MP infusions.

We measured the total number of T-cells, CD4+ T helper cells, CD8+ cytotoxic T-cells, B cells, NK cells and the level of interferon alpha over time upon monthly MP infusions (fig.3). A very rapid and sustained drop in interferon alpha was observed. At the same time, lymphocyte numbers in the peripheral blood remained more or less the same and cellular function was not dramatically altered in proliferation assays. If these effects of MP were evaluated at the level of lymphocyte counts in the cerebrospinal fluid of this particular patient with Aicardi-Goutières syndrome, once again there is a rapid drop in the concentration of interferon alpha, without dramatic changes, certainly not a reduction in cell number.

The interpretation of these data was that lymphocyte counts in blood and cerebrospinal fluid remained unchanged, whereas the interferon alpha levels steeply dropped upon MP infusions. A decrease in interferon alpha does not simply induce immediate changes in cell counts.

We made a more precise immunophenotyping study of the lymphocytes by comparing blood and cerebrospinal fluid lymphocytes on venapunctures and lumbar punctures every month, starting just before the first MP infusion. There seems to be a selective outgrowth or recruitment of lymphocytes to the cerebrospinal fluid compartment, as indicated by the changed CD4/CD8 ratio. What does that mean? It means that what we found in the cerebrospinal fluid, that most of the cells were T-cells. And most of these T-cells were CD8+ cytotoxic T-cells causing the inversed ratio in CSF fluid in all three patients.

This is clearly different from multiple sclerosis. The same analysis of CSF lymphocytes in adult patients with multiple sclerosis shows an selective increase in CD4+ T cells. Clearly, Aicardi-Goutières syndrome leads to the recruitment of some other cell type. A
little calculation, adding up the CD4+ and CD8+ T cells, indicates the presence of another T cell. Thus, there is a CD3+ T cell type, which does not express CD4 or CD8, a T-cell population which is clearly present in these patients. What do these cells represent then? They are probably not normal CD4 or CD8 cells, so how to characterise these cells and are these cells important in the disease?

Most of the CD3+ and CD4/CD8-negative T cells, what’s possible that they can represent. Dependent on the type of T cell receptor, these cells will most likely recognise different antigens than the ones recognised by normal CD4+ or CD8+ T cells. They contribute to innate immunity, especially to mycobacterial infections, to yeast, and densely capsulated pathogens. As suggested under certain autoimmune conditions, these double-negative T cells cells may be burnt-out CD8+ cytotoxic T cells. Being once CD8+, the cells have simply negated their continuous activation, and resolve their inflammatory characteristics by downregulation of CD8 in order to escape from death or deletion. Also a so-called regulatory NK / T-cell was recently identified and described in a vast number of studies on these cells under normal conditions and especially in autoimmune diseases.

To be honest, we have of course measured the critical antigens to distinguish these subsets of cells, although you must remember that the number of T cells from one spinal tap was only enough to do one or two single stainings. In functional terms, it is far more important that we have generated cell lines from these cerebrospinal fluid taps. Now that we have high numbers of cells for functional studies, we can pose questions about aetiology and pathogenesis.

Are these cells producing or sensitive to interferon alpha? Are these cells functionally active or cytotoxic? What antigen do these cells respond to? Is it that they are responsive to glycolipids presented for instance by astrocytes or microglial cells? Those are the questions that we can pose now and probably answer within a couple of months or a year.

So, the steroid treatment led to a drop in interferon alpha, certainly not to a change in either of these two subpopulations and, whether they are sensitive to interferon alpha or not can be tested now that we have cell lines.

These are the future plans: T cell cloning is being performed, so now we can do far more extensive immunophenotyping and a proper functional characterisation of these cells. When we have done that, we can go back to autopsy material or brain biopsies, to see whether these cells can be found and especially around those areas where, for instance, calcification occur. Important questions since we know so little about the exact pathology.
I will stop here and I hope I have at least explained a little bit about what we did in the last couple of months.