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Introduction

The signalling adaptor protein DAP 12 / KARAP / TYROBP (DNAX activating protein of 12 kDa / killer cell activating receptor-associated protein / tyrosine kinase binding protein) is a transmembrane protein with an immunoreceptor tyrosine-based activation motif (ITAM motif). This adaptor is non-covalently associated with multiple cell-surface activating receptors and mediates signal from an extracellular receptor to the cytoplasm of the cell. Its ITAM motif allows the recruitment and the activation of Syk and ZAP 70 tyrosine kinases and leads to cell activation. DAP 12 was initially described in NK cells and later found associated with several activating immunoreceptors including the KIRs, Ly49s, NKG2C/E, TREMs, SIRP-b1, CD200R, MDL-1 suggesting broad cellular expression.

The aim of this work was to study DAP 12 expression in whole blood cells from healthy donors, in order to establish normal values. A further study will compare them to those found in pathological situations. For that purpose, an anti-human DAP 12 monoclonal antibody was produced and conjugated to Alexa-647 fluorophore. We investigated particularly DAP 12 expression in NK cell sub-populations based on KIR expression (NK KIR repertoire).

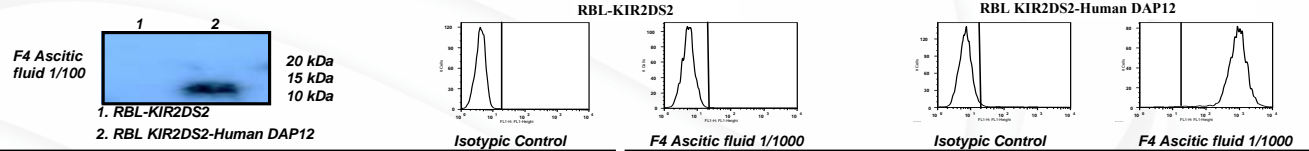
Materials and methods

Whole-blood samples from 10 healthy donors were collected and stained in a first step with several surface markers (as described below). The blood was then treated with a permeabilization reagent (IntraPrep™ permeabilization reagent, IM2388) and stained with DAP-12-Alexa 647-conjugated antibody. Leukocyte subsets were analyzed as follows :

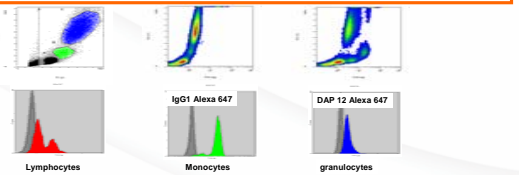
- **NK cells** : CD3-/CD56 bright/CD16- and CD3-/CD56 dim/CD16+
- The NK combination was associated with each of the following NK receptors : NKP30, NKP44, NKP46, CD158b, CD158e, CD158i, CD158a, NKG2D
- **NKT lymphocytes** : CD56+ / CD3+
- **T lymphocytes** : CD3+/CD4+ and CD3+/CD8+
- **Memory T lymphocytes** : CD4+/CD45RA+
- **B lymphocytes** : CD19+ / CD3+
- **Monocytes** : CD14+ / CD33+
- **Neutrophils** : CD16+ / CD16+ / ILT3+

Generation and Characterization of anti-human DAP 12 monoclonal antibody

We generated monoclonal antibodies against the intracytoplasmic tail of the DAP12 protein. The immunisations were performed on Lou rats. The serum immuno-reactivity was tested by ELISA. Splenocytes of immunized rats were used for the fusion with the X63 myeloma cell line. The Ig-F4 secretory hybridoma were selected on HAT selective medium. Culture supernatants were tested again by ELISA . The positive hybridoma were then cloned by limiting dilution. We selected the F4 clone which is a rat IgG1 isotype. Specificity of the generated antibody was tested on a DAP12 transfected cell line RBL-KIR2DS2-human DAP12 and a non transfected cell line RBL-KIR2DS2 by Western Blot or flow cytometry.

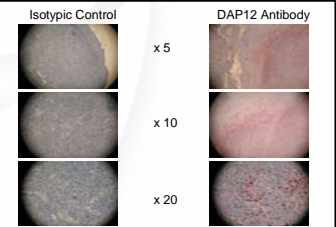


Evaluation of DAP 12 expression by flow cytometry on the three major leukocyte sub-populations

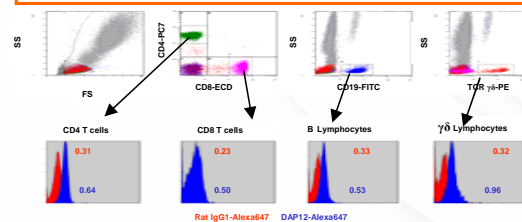


Human lymph node paraffin embedded tissues stained with anti-DAP 12 mAb

These pictures show lymph node paraffin sections stained with either isotypic control (left) or DAP 12 antibody (right). These antibodies were revealed with Histostain Plus Kit (Zymed). We can observe a clear DAP 12 staining of the section more pronounced at the periphery of the lymph nodes.

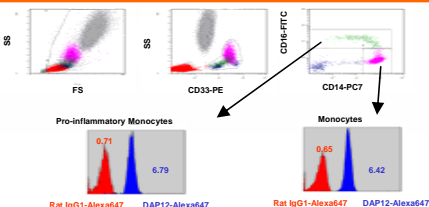


Analysis of DAP 12 expression on lymphocyte sub-populations

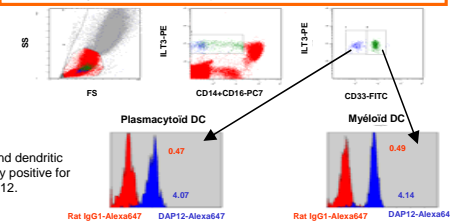


We can observe a slight expression of DAP 12 compared to the isotypic control. This will be confirmed by RT-PCR.

Analysis of DAP 12 expression on monocyte sub-populations



Analysis of DAP 12 expression in dendritic cells

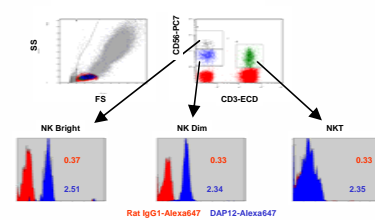


Monocytes and dendritic cells are clearly positive for DAP 12.

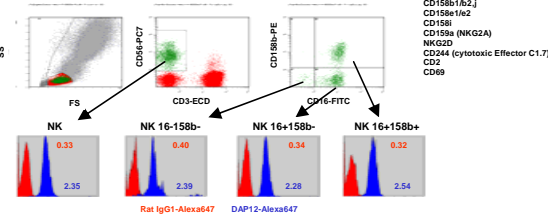
Analysis of DAP 12 expression on leukocyte subsets and NK cells

Analysis of DAP 12 expression among the NK repertoire

NK cells are lymphocytes of the innate immune system, which recognize and eliminate microbe-infected cells, tumor and allogeneic cells. NK cells are regulated by surface receptors with either activating or inhibitory functions. Some of those receptors are associated with DAP 12 adaptor. It was demonstrated that DAP 12 expression is decreased in autoimmune diseases like systemic lupus erythematosus. Toyabe et Al. showed that DAP 12 protein and mRNA expression were decreased in NK cells from lupus erythematosus patients compared to healthy donors. Our analysis first allows to provide the expression pattern of DAP12 expression by flow cytometry among the NK repertoire in healthy donors.



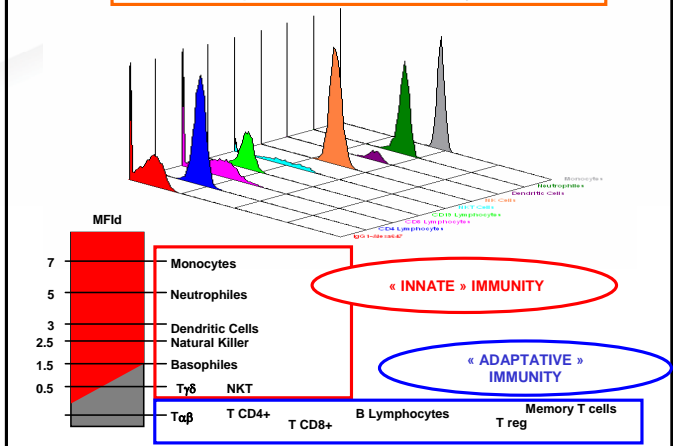
In this analysis, we assessed the pattern expression of DAP12 among the NK repertoire. We performed five color combinations, with all the NK repertoire markers conjugated with Phycoerythrin. We conclude that all the NK sub populations express DAP 12 at the same level.



Results and discussion

The results obtained with the five color combinations showed that DAP 12 protein expression is restricted to the innate immune system. DAP 12 was found to be expressed in neutrophils, monocytes, dendritic cells and NK cells in agreement with previous transcriptional analyses. Our results showed that DAP12 is probably slightly expressed in the cells of the adaptive immune system. This work allows us to define normal values of DAP 12 expression in leukocyte subsets and more specifically among the different NK subsets. The next step is the analysis of the DAP 12 protein pattern expression in pathological situations. Indeed, DAP 12 was found to be involved in several diseases such as infectious (sepsis), autoimmune (lupus and rheumatoid arthritis) and inflammatory diseases. It will be very interesting to look at the up- or down-regulation of this marker in such pathologic situations. In summary, we have analyzed for the first time, the expression pattern of the DAP 12 protein on leukocyte subsets of the innate and adaptive immunity systems.

DAP 12 expression in the different leukocyte subsets



References

- E. Tomasello et Al. Eur. J. Immunol. 2005. 35: 1670-1677.
- S. Toyabe et Al. J. Autoimmunity. 2004. 23 : 371-378.
- Green et Al. Clinical and Experimental Immunol. 2005.141 : 165-173.

Conclusion

We have generated a rat monoclonal antibody against the DAP 12 protein. The F4-Alexa-647 conjugated antibody permitted the analysis of the expression pattern of the DAP 12 protein on leukocyte subsets. We confirmed the results published at the mRNA level. We have demonstrated that DAP 12 is strongly expressed to cells of the innate immune system.