4813: Problem-based Learning Workshop

Advanced Laboratory studies for Primary Immunodeficiency Disorders

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Discussion Leader: Roshini Abraham, PhD

- Understand B cell flow analysis in CVID (PBL case)
### MARKERS FOR PERIPHERAL B CELL SUBSETS - CURRENT

<table>
<thead>
<tr>
<th>Subset</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total B cells</td>
<td>CD19 and/or CD20</td>
</tr>
<tr>
<td>Transitional B cells</td>
<td>CD19+CD38+IgM+</td>
</tr>
<tr>
<td>Total IgM+ B cells</td>
<td>CD19+IgM+ (includes naïve B cells)</td>
</tr>
<tr>
<td>Memory B cells</td>
<td>CD19+CD27+</td>
</tr>
<tr>
<td>Switched memory B cells</td>
<td>CD19+CD27+IgM-IgD-</td>
</tr>
<tr>
<td>Marginal zone B cells</td>
<td>CD19+CD27+IgM+IgD+</td>
</tr>
<tr>
<td>IgM-only memory B cells</td>
<td>CD19+CD27+IgM+IgD-</td>
</tr>
<tr>
<td>Plasmablasts</td>
<td>CD19+CD38+IgM-</td>
</tr>
<tr>
<td>CD21+ B cells</td>
<td>CD19+CD21+</td>
</tr>
<tr>
<td>CD21- B cells</td>
<td>CD19+CD21-</td>
</tr>
</tbody>
</table>

- With newer information, more suitable cellular markers are available for accurate identification of transitional B cells and plasmablasts, in particular, but also for naïve B cells.
## NEWER B CELL MARKERS FOR B CELL SUBSET QUANTITATION

Total B cells and B cell subsets can be quantitated in blood using multicolor flow cytometry:

<table>
<thead>
<tr>
<th>Total B cells:</th>
<th>CD45+CD19+20+</th>
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</table>

For B cell subset analysis, the gating strategy uses CD45+19+20+/- (depending on the subset being studied), thus these markers are not specifically repeated in the panel below:

### Transitional B cells:

- **T1:** \( CD24^{hi}38^{hi}10^{+}27-21^{\text{low}} \text{IgM}^{+++} \)
- **T2:** \( CD24^{hi}38^{hi}10^{+}27-21^{\text{int}} \text{IgM}^{+++} \)

### Naïve B cells:

\( \text{IgM}^{+}\text{IgD}^{+}27-38-21^{+++} \)

### Memory B cells:

- **Marginal zone B cells:** \( \text{CD27}^{+}\text{IgM}^{+}\text{IgD}^{+} \)
- **IgM-only memory:** \( \text{CD27}^{+}\text{IgM}^{+}\text{IgD}^{-} \)
- **IgD-only memory:** \( \text{CD27}^{+}\text{IgM}^{-}\text{IgD}^{+} \)
- **Class-switched memory:** \( \text{CD27}^{+}\text{IgM}^{-}\text{IgD}^{-} \) (IgG+ or IgA+ or IgE+)
<table>
<thead>
<tr>
<th></th>
<th>CD19+20-IgD-IgM-CD38++27+138+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmablasts:</strong></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td><strong>Assessment of CD21</strong></td>
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<tr>
<td><strong>expression on B</strong></td>
<td></td>
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<tr>
<td><strong>cells:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>CD21+:</strong></td>
<td>CD19+20+21+</td>
</tr>
<tr>
<td><strong>CD21−:</strong></td>
<td>CD19+20+21−</td>
</tr>
<tr>
<td><strong>CD21^{low}:</strong></td>
<td>CD19+20+21^{low}</td>
</tr>
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</table>
EXAMPLES OF B CELL SUBSET ANALYSIS

Healthy Donor

- **CD19+CD20+ total B cells**
- **CD19+CD27+ (memory B cells)**
  - CD19+CD27+ IgM+IgD+ (marginal zone B cells)
  - CD19+CD27+ IgM+IgD- (IgM-only memory B cells)
- **CD19+CD27+ IgM-IgD- (switched memory B cells)**
CD19+CD38++IgM+ defined as transitional B cells in EUROclass and Freiburg schemes

CD19+CD38++IgM- defined as plasmablasts in EUROclass and Freiburg schemes

- Other laboratories may define the entire upper right quadrant as transitional B cells and the entire upper left quadrant as plasmablasts without differentiating between intensity of expression of CD38 on B cells in conjunction with presence or absence of IgM
- This could result in quantitative variability for these B cell subsets between clinical laboratories depending on gating strategy

Healthy Donor → CVID Patient
PBL CASE: B CELL SUBSET ANALYSIS

- Decreased total memory and switched memory B cells, expansion of CD21^{dim/low} B cells
OTHER EXAMPLES OF ABNORMAL B CELL SUBSETS IN CVID PATIENTS

Patient A

- Decreased total memory and switched memory B cells, expansion of CD21\textsuperscript{dim/low} B cells (pediatric CVID patient)

Patient B

- Increased total IgM\textsuperscript{+} B cells with a population having bright IgM expression (in red circle, panel A), decreased total memory and switched memory B cells, unusual and large increase in IgM-only memory B cells (panel C, in red circle), all CD21\textsuperscript{+} B cells are of the CD21\textsuperscript{dim/low} phenotype (panel D- see comparison with healthy donor example for CD21\textsuperscript{+} B cells (pediatric CVID patient)}
**SUMMARY OF PARIS AND FREIBURG B CELL SUBSET CLASSIFICATION FOR CVID PATIENTS AND CLINICAL CORRELATION**

<table>
<thead>
<tr>
<th>Paris scheme (2003)</th>
<th>MB0 48%</th>
<th>MB1 43%</th>
<th>MB2 9%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;11% CD27+ Memory B cells</td>
<td>&gt;11% CD27+ Memory B cells</td>
<td>Not MB0 or MB1</td>
<td></td>
</tr>
<tr>
<td>&lt;8% switched memory B cells (CD27+IgM-IgD-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD19+CD21&lt;sub&gt;low/dim&lt;/sub&gt; B cells &gt;20%</td>
<td>% CD19+CD21&lt;sub&gt;low/dim&lt;/sub&gt; B cells &lt;20%</td>
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<table>
<thead>
<tr>
<th>CVID patients</th>
<th>Paris/Freiburg</th>
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</thead>
<tbody>
<tr>
<td>N = 57</td>
<td>47%/ 58%*</td>
</tr>
<tr>
<td>splenomegaly</td>
<td>34%/41%</td>
</tr>
<tr>
<td>lymphadenopathy</td>
<td>23%/19%</td>
</tr>
<tr>
<td>granulomas</td>
<td>17%/17%</td>
</tr>
<tr>
<td>autoimmune cytopenias</td>
<td>0%/4%</td>
</tr>
<tr>
<td></td>
<td>23%/19%</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Freiburg scheme (2002)</th>
<th>la</th>
<th>lb</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Switched memory B cells (CD27+IgM-IgD-)</td>
<td>Switched memory B cells (CD27+IgM-IgD-)</td>
<td>Switched memory B cells (CD27+IgM-IgD-)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.4% of PBLs (lymphocytes)</td>
<td>&lt;0.4% of PBLs (lymphocytes)</td>
<td>&gt;0.4% of PBLs (lymphocytes)</td>
<td></td>
</tr>
<tr>
<td>% CD19+CD21&lt;sub&gt;low/dim&lt;/sub&gt; B cells &gt;20%</td>
<td>% CD19+CD21&lt;sub&gt;low/dim&lt;/sub&gt; B cells &lt;20%</td>
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</tbody>
</table>

*Associations with clinical phenotypes were found to be statistically significant by the authors’ of both classifications

Warnatz K et al, Blood, 2002, 99: 1544-1551
SUMMARY OF EUROclass (2008)
CLASSIFICATION FOR CVID PATIENTS AND CLINICAL CORRELATION

CVID patients
N = 303 (after removal of Grp. B-)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Total</th>
<th>Grp. B-</th>
<th>Grp. B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenomegaly</td>
<td>41%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>26%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulomas</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoimmune cytopenias</td>
<td>20%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

>1% B cells (grp. B+)

<2% switched memory B cells (CD27+IgM-IgD-) smB-

<=1% B cells (grp. B-)

> =2% switched memory B cells (CD27+IgM-IgD-) smB+

>=9% Transitional B cells (CD19+CD38hiIgMhi) smB-Trhi

<9% Transitional B cells (CD19+CD38hiIgMhi) smB-Trnormal

42% of total patients
58% of total

*Associations with clinical phenotypes were found to be statistically significant

Wehr C et al, Blood, 2008, 111: 77-85
EUROclass (2008) CLASSIFICATION (CONT'D.)

Key B cell subsets in EUROclass scheme: class-switched memory B cells, transitional B cells and CD21^{low/dim} B cells

Wehr C et al, Blood, 2008, 111: 77-85

*Associations with clinical phenotypes were found to be statistically significant
OTHER STUDIES USING B CELL SUBSET ANALYSIS FOR CLASSIFICATION OR CLINICAL “BINNING” OF CVID PATIENTS

1. Detkova D et al (Chest, 2007, 131: 1883-1889): (n = 41) Association between memory B cell reduction and lung disease as well as intestinal involvement (uses the Paris classification) – statistically significant correlation between CVID pts with chronic lung disease (bronchiectasis and reduced FVC+/−FEV1) and reduced total memory B cells and low class-switched memory B cells compared to those with normal memory B cell count
   - Malabsorption syndrome/chronic non-infectious diarrhea in CVID pts with low total memory B cells and decreased class-switched memory B cells compared to those with normal memory B cell count


3. Sanchez-Ramon S et al (Clin Immunol, 2008, 128: 314-321): (n = 105) Decreased class-switched memory B cells identified as an independent risk factor for granulomas, autoimmunity and splenomegaly in CVID. Gender association documented with fewer switched memory B cells in males compared to female CVID pts. Lower baseline IgG was independent predictor of pneumonia and severe infections in this group

LITERATURE REVIEW FOR B CELL SUBSET ANALYSIS AND CLINICAL CORRELATION IN CVID (CONT'D.)

5. Huck K et al (Clin Immunol, 2009, 131: 50-59): (n = 16) Reduced class-switched memory B cells is an useful additional marker for identification of children with CVID (hypogammaglobulinemia) and may be an early marker for CVID onset

6. Al Kindi M et al (Clin Exp Immunol, 2012, 167: 275-281): (n= 53) Attempted to validate previous 3 classifications in their cohort. CVID patients had lower total memory and switched memory B cells compared to controls. Only clinical correlation that could be verified was association of Grp Ia Freiburg to increased prevalence of granulomatous disease. Autoimmune disease correlation could not be verified and in this cohort, EUROclass scheme was not predictive of clinical phenotypes. Significant correlation between low absolute total B cells and total memory B cells with granulomatous disease. Authors suggest absolute quantitation of B cell subsets may be more useful clinically (risk of granulomatous disease and possible autoimmunity) than % data

7. Kutukculer N et al (J Clin Immunol, 2012, 32: 1165-1179): (N= 25) Correlation between 3 CVID B cell classifications (Paris, Freiburg and EUROclass) in 25 Turkish patients. Class-switched memory B cells significantly lower in CVID patients compared to controls and lower in patients with severe disease compared to moderate disease. Severe disease includes patients with splenomegaly, granulomatous disease +/- bronchiectasis and lower baseline IgG (<270mg/dL). Freiburg classifn:- 87.5% in Grp I (75% Grp Ib and 14% in Grp 1a) and 12.5% in Grp II. Significantly lower IgG and IgA in Grp 1a is a novel finding. Paris classifn :- 88% MB0, 4%, MB1 and 8%, MB2. Significant correlation with splenomegaly, lymphadenopathy and autoimmune cytopenias in MB0. EUROclass classifn:- 45.8% smB+ and 54.2%, smB-. Significant correlation with splenomegaly and lymphadenopathy in smB-. B cell subset correlations not helpful in predicting underlying genetic basis for CVID patients
DIAGNOSTIC CRITERIA FOR CVID

- ESID/PAGID criteria (Conley et al, 1999) for diagnosis of CVID: Hypogamma with low IgG +IgA and/or IgM, 2SD below mean of age (probable CVID)

- Low IgG or IgA or IgM below 2 SD for age (possible CVID)

- Onset of immunodeficiency >2 years of age

- Impaired functional antibody responses to antigens (vaccines or isohemagglutins)

- Exclusion of other defined causes of hypogamma

SUGGESTED ALTERNATIVES:

- Increase age of diagnosis from 2y to 4y (Cunningham-Rundles, 2010) to exclude other causes of hypogamma, particularly THI

- IgG cutoff levels of 2SD for age does not take into account that most labs use a 95% confidence interval, therefore, 2.5% healthy individuals will fall below the lower limit of most reference ranges (and 2.5% above the upper limit)

- Alternate cutoff, based on data from European (Chapel H et al, 2008) and American (Cunningham-Rundles C et al, 1999) cohorts suggest that 450 mg/dL may be reasonable since the majority of patients in both groups had less than this level at diagnosis

- Tiered system suggested by Cunningham-Rundles et al (2010) based on amount of IgG present (<150, 150-250, 250-450 and 450-600 mg/dL)

- Further evaluation would be based on the IgG levels, e.g. vaccine antibody responses would not be evaluated in patients with IgG <150 mg/dL; higher IgG levels would require more evaluation, especially for vaccine responses, and in patients with IgG between 450 and 600 mg/dL with close to normal IgA, functional antibody responses are likely to be intact

- Patients in this latter category may need regular assessment as their immunological status may change over time leading to requirement for Ig replacement, and this may or may not be accompanied by change in clinical status

- Recurrent or significant infections is not a mandatory requirement because while the majority will fall into this category, a subset of patients, such as the case presented herein, will present first with autoimmunity, or granulomatous disease (6% of patients in a large cohort of 473 patients did not have infectious complications, Resnick E et al, Blood, 2011)
CLINICAL RELEVANCE OF B CELL SUBSET ANALYSIS IN CVID

- B cell subset analysis, may not be as valuable in diagnosis, due to CVID heterogeneity, as it may be in classification and potentially, prognosis

- Accurate classification of disease essential to optimal management

- B cell subset analysis, based on published data, may provide insight into risk for development of other pathological features, autoimmune cytopenias, granulomatous disease etc in CVID patients

- Classification/stratification of CVID patients is likely to allow differentiation of patients who have shared patho-physiological basis of disease and shared features of response to treatment, and these two may not overlap in some patients

- The genetic heterogeneity (of the known monogenic defects and the unknown likely polygenic defects) of CVID is indicative of the “many roads lead to Rome” observation that there can be substantially different immunological anomalies all resulting in hypogammaglobulinemia, as a common phenotype

- B cell subset analysis should probably not be used as a level 1 test for a diagnostic work-up for CVID

- Once a diagnosis of CVID has been established (based on previously defined criteria) it may be reasonable to evaluate B cell subsets to determine immunological classification of patient and when possible, correlation with clinical phenotype and immunoglobulin levels

- The value of longitudinal analysis remains unclear; the clinical and immunological phenotype of CVID may be an evolving process, and changes in B cell subsets over time may be useful and indicative of disease evolution (patient presented here had normal switched memory B cells initially but developed a notable decrease in this subset over time with expansion of CD21^{dim/low} B cells

- Serial monitoring, if performed, should be undertaken in the same laboratory so as to permit consistent interpretation of results
# GENE DEFECTS IN CVID AND B CELL SUBSET ANALYSIS

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>B cell phenotype</th>
</tr>
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<tbody>
<tr>
<td><strong>CD19</strong></td>
<td>Normal total B cells with absent CD19 but normal CD20 expression, low class-switched memory B cells</td>
</tr>
<tr>
<td><strong>CD21</strong></td>
<td>Normal total B cells, low class-switched memory B cells</td>
</tr>
<tr>
<td><strong>CD81</strong></td>
<td>Normal total B cells with absent CD19 (since CD81 is required for CD19 expression but normal CD20 expression, low class-switched memory B cells</td>
</tr>
<tr>
<td><strong>CD20</strong></td>
<td>Normal total B cells, absent CD20 expression, decreased class-switched memory B cells, impaired polysaccharide antibody response</td>
</tr>
</tbody>
</table>

- All of the above defects affect B cell activation and Ca²⁺ flux in B cells. Ca²⁺ flux is also low in B cells of patients with low class-switched memory and expanded CD21dim/low B cells

| **ICOS**    | B cell numbers can be reduced (especially adults) but may be elevated (in children), naïve B cells are normal, decreased class-switched memory B cells |
| **TNFRSF13B (TACI)** | Likely a disease-associated than disease-causing, at least in the heterozygous state, rarely severe B cell lymphopenia, reduced switched memory B cells in at least 2/3rd of patients, other disease-modifying factors may influence immunological and clinical phenotype, autoimmunity and lymphoproliferation observed at higher frequency |
| **TNFRSF13C (BAFF-R)** | B cell lymphopenia, increased transitional B cells, decreased marginal zone B cells and class-switched memory B cells |

- Other SNPs (especially in DNA repair genes) and linkage to genes in MHC locus have been described to be associated with CVID phenotype

- Genome-wide linkage study (GWAS) demonstrated copy number variations (CNV) in majority of a 363 patient cohort – Orange et al, JACI, 2011)
TAKE-HOME POINTS WITH REGARD TO B CELL SUBSET ANALYSIS IN CVID

1. Several studies in literature with regard to B cell subset analysis and potential clinical correlations in CVID

2. Studies support a correlation between reduced total memory B cells +/- switched memory B cells in CVID and possible adverse phenotype compared to CVID patients with normal memory B cells

3. Clinical correlations thus far are not perfect and therefore, B cell subset analysis has little value in making a diagnosis of CVID

4. However, may be potentially useful in prognosis and classification though no definitive correlations have been established with survival and outcome

5. Low class-switched memory B cells also seen in Hyper IgM syndromes (CD40L and CD40 deficiency) as well as other PIDs, such as WAS, Hyper IgE syndrome, CGD but has no direct clinical value in establishing diagnosis for these other PIDs. Other disease-specific and more relevant tests are available

6. Expansion of CD21dim/low B cells appears to have unique pathogenic role and suggests differences in underlying immunological defects though clinical correlations are still not fully defined

7. One study (Carsetti et al, JACI, 2005, 115: 412-417) suggested that absence of IgM+ memory B cells in CVID patients was associated with increased risk for recurrent bacterial pneumonia and bronchiectasis. Findings not verified in other CVID classification studies

8. Analytical variability in clinical labs can influence individual cut-offs for B cell subsets. However, overall theme of low total memory or class-switched memory B cells in CVID patients and potential for clinical complications is irrespective of lab-specific cut-off

9. Several clinical labs offer some version of B cell subset analysis – most typically quantify memory B cell components (switched memory B cells) but do not necessarily provide additional granular analysis (CD1dim/low etc)
TAKE-HOME POINTS (CONT'D.)

10. Pediatric reference values have to be used for interpreting B cell subset data in children. Adult ranges for memory B cells or memory B cell subsets (switched memory B cells, marginal zone B cells etc.) cannot be used to define normal and abnormal frequencies or absolute counts in children (Smet J et al, Clin Immunol, 2011, 138: 266-273, Piatosa B et al, Cytometry Part B, 2010, 78B: 372-381)

11. When interpreting laboratory data and attempting to perform clinical correlations based on literature, it is important to know which markers were used to define specific B cell subsets so that reasonable comparative correlations can be made relative to uniform B cell panels

12. CVID is immunologically and genetically heterogeneous and at present, no clear correlations between specific patterns of B cell subsets are associated with specific genotype. Therefore, B cell subset analysis cannot be used to identify potential genetic defect

13. Defects in B cell subsets may evolve over time and therefore longitudinal analysis may be revealing and provide prognostic value, however, these correlations are lacking at present in the literature

14. Additional data required to establish correlations between specific changes in B cell subsets, e.g. memory B cells in CVID and clinical outcomes and survival. Such data may be extrapolated from current studies but may not always be completely valid

15. T cell defects also observed in subset of CVID patients – future clinical stratification may be more robust with inclusion of both T and B cell subset phenotyping

16. Other clinical uses of B cell subset analysis include evaluation of B cell reconstitution post-HCT, recovery of switched memory B cells post-B cell-depleting therapy, which has been shown to coincide with relapse of disease in RA, SLE, pSS and certain neurological autoimmune diseases
TREC AND KREC ANALYSIS IN CVID: CORRELATION WITH CLINICAL OUTCOME/SURVIVAL (A NEW CLINICAL CLASSIFICATION FOR CVID?)


- TREC = T cell receptor excision circle; identifies naïve T cells and represents thymic output

- KREC = kappa rearrangement excision circle, can also be called B cell receptor excision circle and identifies production of naïve B cells

- n = 40 CVID patients, patients classified into 4 groups, A-D based on TREC/KREC copy numbers, clinical complications highest in group D patients and least in group A. Complications included malignancy, autoimmunity and opportunistic infections

- No significant correlations found between any of the B cell subset classification schemes and clinical events in any group (A-D)

- Cumulative clinical events significant different between groups A, B, C and D between each other

- TREC/KREC-based classification matches clinical outcomes and may facilitate better diagnostic labeling of patients, especially those with T and B cell defects

- Group A patients had fewest clinical events and suggest that CVID patients without T cell defects may have better outcomes than those with T cell defects

- Autoimmunity frequently seen in Group B patients, though heterogeneity may be present in this group with regard to T cell phenotype

- TREC/KREC may be useful for assessment of clinical severity and differentiation of patients with Combined Immunodeficiency from CVID, who may require a different treatment approach
Bibliography for B cell subset analysis in CVID:

5. The EUROclass trial: defining subgroups in CVID. Wehr C et al, Blood, 2008, 111: 77-85
6. B cell receptor-mediated Ca2+ signaling is impaired in B cells of Type 1a patients with CVID. Foerster C et al, J Immunol, 2010, 184: 7305-7313
13. Severe deficiency of switched memory B cells (CD27+IgM-IgD-) in subgroups of patients with CVID: a new approach to classify a heterogeneous disease (Freiburg classification). Warnatz K et al, Blood, 2002, 99: 1544-1551
15. Memory switched B cell percentage and not serum immunoglobulin concentration is associated with clinical complications in children and adults with specific antibody deficiency and CVID. Alachkar H et al, Clin Immunol, 2006, 120: 310-318
20. CVID patients with increased CD21-/low B cells suffer from altered receptor editing and defective central B cell tolerance. Romberg N et al, Blood, 2011, 118: 5977-5978
22. Patients with CGD have a reduced peripheral blood memory B cell compartment. Blessing JJ et al , J Immunol, 176: 7096-7103