Deciphering the molecular bases of the biological effects of antibodies against Interleukin-2: A versatile platform for fine epitope mapping

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ABSTRACT

Elucidating the network of interactions established by Interleukin-2 is a key step to understanding its role as a master regulator of the immune system. Binding of this cytokine by specific antibodies gives rise to different classes of immune complexes that boost or inhibit immune responses. The molecular bases of such functional dichotomy are likely related to the nature of the recognized epitopes, making it necessary to perform fine epitope mapping studies. The current work was aimed at developing a versatile platform to do so. This was accomplished by display of human and mouse Interleukin-2 on filamentous phages, together with extensive mutagenesis of both antigens and high throughput screening of binding properties of more than 200 variants. Detailed molecular pictures of the epitopes were thus delineated for four antibodies against either human or mouse Interleukin-2, which refined and, in some cases, modified the conclusions derived from previous mapping studies with peptide libraries. Overlapping surface patches on mouse Interleukin-2 that also coincide with the predicted interface between the cytokine and its receptor alpha chain were shown to be recognized by two monoclonal antibodies that promote enhancement of immune responses, shedding new light on the structural bases of their biological activity. Our strategy was powerful enough to reveal multiple binding details and could be used to map the epitopes recognized by other antibodies and to explore additional interactions involving Interleukin-2 and related cytokines, thus contributing to our understanding of the complex structure–function relationships within the immune system.

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Introduction

Interleukin-2, originally characterized as a T cell growth factor (Smith 1980), is now considered to be a master regulator of immune function, controlling the delicate balance between immune responsiveness and tolerance (Malek 2008). This view is supported by its multiple (and often paradoxical) roles in inflammatory and regulatory phenomena underlying immunity (Malek and Bayer 2004; Hoyer et al. 2008).

Such functional dichotomy has attracted interest in targeting IL-2 and its receptor for therapeutic purposes in different settings (Rosenberg and Lotze 1986; Manoukian and Hagemeister 2009). Attempts to modulate IL-2 functions through the use of anti-IL-2 antibodies have given rise to definition of two classes of antibodies: those that boost immune responses and those that inhibit them (Boyman et al. 2006). Immune complexes formed by different antibodies have been used to enhance responses to anti-viral vaccination (Mostböck et al. 2008) or to induce tolerance in the context of pathological autoimmunity and organ transplantation (Webster et al. 2009).

Putative IL-2 epitopes recognized by each antibody class have been proposed on the basis of functional studies and theoretical analysis of the consequences of selectively blocking interactions with the different receptor chains (Boyman et al. 2006). However, the precise molecular details of the epitopes remain unknown, since no mapping experiments have been reported for these antibodies. Some efforts have been made to map other anti-IL-2 antibodies with phage-displayed random peptide libraries (Vispo et al. 1999). Linear peptides from a random library (and even those derived from the antigen itself) do not always mimic the interactions established by the original Ag, particularly when the target

epitope is discontinuous (Deroo and Muller 2001). Even when peptides resembling Ag sequences are identified, molecular contacts can be different as compared to recognition in the context of the native protein (Saphire et al. 2007; van Regenmortel 2009).

The current work aimed to develop a mapping strategy to dissect the epitopes directly on both human and mouse IL-2 molecules. This goal was accomplished through phage display of antigenically reactive IL-2 molecules of both species. Scanning their surfaces through extensive mutagenesis/randomization rendered a collection of more than 200 variants that were screened in a high throughput manner to detect either loss or gain of recognition by antibodies. The strategy was useful to define the epitopes recognized by four mAbs. This approach could be extended to map epitopes for other anti-IL-2 antibodies and to reveal structural insights of additional interactions involving IL-2, thus contributing to our understanding of the complex structure–function relationships within the immune system.

Materials and methods

IL-2 phage display

Genes coding for human or mouse IL-2 (flanked by ApaLI and NotI restriction sites) were synthesized by GENEART (Germany), using codons optimized for *Escherichia coli* expression. Synthetic genes were cloned into the pCSM phagemid vector that includes the sequence coding for DsbA signal peptide (MKKIWLALAGLVLAF-SASA) (Steiner et al. 2006). TG1 *E. coli* cells (K12 (*lac-pro*), *sup*E, *thi*, *hsd*D5/F' *tra*D36, *pro*A⁺B⁺, *lac*I�q, *lac*Z M15) were transformed with the resulting genetic constructs and used to rescue phages displaying IL-2 molecules fused to the gene-3 minor coat protein (P3) on a 50 ml scale (Marks et al. 1991). Specific recognition of phage-displayed molecules was tested by phage ELISA (see below).

Site-directed mutagenesis and randomization

Protein sequences of phage-displayed human and mouse IL-2 molecules were aligned to detect differences between them (Fig. 1) Solvent-exposed residues (>20%) were located on the hIL-2 structures contained in PDB files 2B5I and 2ERJ. A panel of phage-displayed IL-2 variants was obtained (Table 1) by individually replacing every solvent-exposed residue by the aa found at the equivalent position in the protein of the other species (Fig. 1). mIL-2 was also modified by deleting two fragments that have no counterpart in hIL-2. Additional variants were obtained by introducing multiple simultaneous mutations or by randomizing selected positions. All the variants were constructed by site-directed mutagenesis (Kunkel 1985), using described procedures (Fellouse and Sidhu 2007). Briefly, single strand DNA (from hIL-2/mIL-2 gene-containing pCSM phagemid) obtained from phages produced by the CJ236 *E. coli* strain (*dut⁻ ung⁻ thi*-1 *relA1 spoT1 mcrA*/pCJ105 (F' *cam*ʳ)) was used as template. Antisense mutagenic oligonucleotides included 15 nucleotides complementary to the template at each end, flanking the region to be modified. Such target region contained one or more triplets to be changed in order to obtain the desired mutation(s). Randomization was accomplished by inserting the degenerate triplet NNK at a given codon position.

Modeling mIL-2 structure

The aa sequence of mIL-2 (code P04351) was retrieved from UniProt (Jain et al. 2009; Consortium 2011). The first 20 residues, corresponding to the signal peptide, were removed. The remaining 149 aa were used to perform a BLAST (Altschul et al. 1990), as implemented by UniProt, against the PDB (Bernstein et al. 1977), in order to find suitable structural templates for the model. Since only hIL-2

resulted in a significant match, 13 structures containing the hIL-2 molecule were downloaded from the PDB (codes 1M47, 1M48, 1M49, 1M4A, 1M4B, 1M4C, 1NBP, 1PW6, 1PY2, 1QVN, 1Z92, 2B5I, and 3INK) to be used as templates.

A multiple alignment was performed, using the software T-COFFEE 5.72 (Notredame et al. 2000), between mIL-2 sequence and hIL-2 sequences corresponding to downloaded templates. Alignment results and template structures were used as input for the program MODELLER 9v7 (Sali and Blundell 1993) to generate the homology model of mIL-2. Quality of the model was evaluated with the built-in MODELLER functions. The region comprising the first 22 aa was modeled poorly due to the lack of corresponding residues in the template structures (17 mIL-2 residues within this region have no structural equivalents in hIL-2).

Phage screening by ELISA

TG1 *E. coli* cells were transformed with IL-2-containing genetic constructs or mutagenesis reaction products. Colonies were used to rescue IL-2 displaying phages in a 96-well format (Marks et al. 1991). Polyvinyl chloride microplates were coated overnight at 4 °C with either anti-IL-2 mAbs, the anti-*c-myc* tag 9E10 mAb or the unrelated mAb 1E10, at 10 µg/ml in PBS. Plates were blocked for 1 h at RT with PBS containing 4% skim powder milk (M-PBS). Phage-containing supernatants from 96 deep-well plates (diluted 1/5 in M-PBS), or purified phages (also diluted in M-PBS), were added. After 2 h at RT, plates were washed with PBS containing 0.1% Tween 20 and an anti-M13 mAb conjugated to horseradish peroxidase (GE Healthcare, USA), appropriately diluted in M-PBS, was added. Plates were incubated for 1 h at RT and washed. Substrate solution (500 µg/ml *ortho*-phenylenediamine and 0.015% hydrogen peroxide in 0.1 mol/l citrate-phosphate buffer, pH 5.0) was added. The reaction was stopped after 15 min, with 2.5 mol/l sulfuric acid. Absorbances were measured at 492 nm.

Molecular characterization of selected IL-2 variants

Clones producing phage-displayed IL-2 variants that conserved, lost, or gained recognition by one or more anti-IL-2 mAbs were selected for further characterization. Only those IL-2 variants that were successfully displayed (as assessed by ELISA with the anti-*c-myc* tag 9E10 mAb) were included in the analysis. XL1-Blue *E. coli* cells (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* F' *pro*AB *lac*IqZ M15 Tn10 Tetʳ) were infected with the corresponding phage-containing supernatants and used to purify plasmid DNA with the QIAprep Spin Miniprep kit (Qiagen, USA). Phagemid inserts were sequenced by Macrogen (Korea).

Results

Both human and mouse IL-2 were displayed on filamentous phages

Display of human and mouse IL-2 proteins on filamentous phages was shown by recognition of viral particles by an antibody against the *c-myc* peptide (a tag fused to the C terminus of the heterologous protein on the phage surface in our display system) (Fig. 2). Beyond the mere presence of phage-displayed IL-2 molecules, their proper antigenicity was confirmed by their reactivity with a panel of specific monoclonal antibodies: IL-2.1 and IL-2.2 mAbs for hIL-2 (Vispo et al. 1999); S4B6, JES6-5H4 and JES6-1A12 mAbs for mIL-2 (Boyman et al. 2006). Correct folding of phage-displayed hIL-2 was additionally shown by its binding to immobilized human CD25 (the IL-2 receptor alpha chain). The above-described results confirmed that phage-displayed hIL-2 and