European Review for Medical and Pharmacological Sciences 2017; 21: 1583-1587

# SUMOylation in cardiac disorders – *a review*

X.-C. LI, Y. ZENG, R.-R. SUN, M. LIU, S. CHEN, P.-Y. ZHANG

*The Affiliated XuZhou Center Hospital of Nanjing University of Chinese Medicine, XuZhou Central Hospital, XuZhou, China*
*The Affiliated XuZhou Hospital of Medical College of Southeast University XuZhou Clinical School of Xuzhou Medical College, XuZhou, China*

**Abstract. – SUMOylation regulates diverse cellular processes including transcription, cell cycle, protein stability, and apoptosis. A recent research has now revealed the role of SUMO1 in cardiac disorders. Studies have evidenced that failing heart induces SUMO2/3 conjugation. Moreover, increased SUMO2/3- dependent modification has been observed to result in congestive heart disease such as cardiac hypertrophy by promoting cardiac cell death. Also, few recent studies have confirmed the role of SUMOylation in cardiac protein degradation. On the other hand, over-expression of SENP5, SUMO2/3-specific deconjugation enzyme has been observed to result in dilated cardiomyopathy or cardiac failure. So, the present review article would enlighten the latest updates about SUMOylation and associated factors during cardiac disorders.**

Key Words
SUMOylation, SUMO2/3, SENP5, Cardiac disorders.

## Introduction

The precise and specific control of proteins is essential for normal homeostasis during the entire life span. Protein modifications viz. PTM are most widely used options for the maintenance of physiologic homeostasis. Further, PTM included covalent attachments of sugars, lipids or chemical groups such as phosphate, acetyl or methyl groups into proteins. These modifications are also critical for spatial as well as temporal modulation of protein function. Ubiquitylation is a prominent protein-based modification, involving protein degradation as a mark to be recognized by the proteasome[1]. Nearly two decades after discovering ubiquitin, SUMO (another Ubiquitin-related modification), has been identified. SUMO stands for Small Ubiquitin-like Modifier. Its unstable attachment to proteins and loss of association during experimental treatments make it difficult to find its existence. Also, most of the SUMOylation-dependent modifications remain in an association with nuclear pore component Ran GTPase activating protein (RanGAP1)[2,3], thereby confirming the inability to pursuit for various targets. SUMO is evolutionally conserved from yeast to mammals and is involved in the reversible covalent attachment to targets. SUMOylation pathway is very similar to its biochemical analog, Ubiquitylation[4,5]. Human cells have four distinct SUMO isoforms: SUMO1-SUMO4[6]. SUMO1 to 3 are ubiquitously expressed. SUMO4 is detected in limited tissues including kidney, spleen and lymph node. However, substrate and function for SUMO4 are still unclear[7]. SUMO isoforms are expressed immaturely, and their C- terminals are usually stretched. Mature isoforms expose diGlycine (Gly-Gly) motif, serving as an active acceptor to attach free amino group (NH2) of lysine via an isopeptide bond.

Mature SUMO isoforms show very similar 3D structures of ubiquitin in spite of less amino acid homology[8]. This similarity causes competition and/or interchange for substrate affinity. Sequential SUMOylation cascade requires three enzymes: an E1 activating enzyme, an E2 conjugating enzyme, and SUMO E3 ligase. The first step is initiated from exposure of C-terminal diglycine motif of SUMO. SAE1/2, SUMO activating enzyme is then able to associate with mature SUMO by consuming ATP, resulting in transferring SUMO to Ubc9, E2 conjugating enzyme. Finally, SUMO is facilitated to conjugate with substrates by E3 ligases including PIAS (Protein Inhibitor of Activated STAT) family, RanBP2, polycomb 2 and MAPL (Mitochondrial-Anchored Protein Ligase)[10]. Compared to ubiquitin pathway, SUMO is conjugated by fewer enzymes[11]. This limitation promoted elevated usage of each SUMO components by distinct cellular occupancy, specificity, and efficient combinatorial coordination. Emerging evidence reported the non-covalent interaction of SUMO isoforms with substrates[12]. So, SIM (SUMO-interacting motif) could also modulate target through conformational chan-

*Corresponding Author:* Peiying Zhang, MD; e-mail: zpying58@126.com

ges[13]. Short hydrophobic peptides of SIM primarily consisting of acidic and/or phosphorylated serine residues govern specificity of SUMO-SIM interactions[14]. Diverse ways of SUMOylation allows SUMO-dependent PTM to regulate target proteins more efficiently.

### deSUMOylation pathway

SUMO-dependent PTM is reversible in nature due to SENPs, Sentrin/SUMO-specific proteases. SENPs are usually identified from cleavage assay within bacterial transformants expressing yeast proteins and sequence homology search using Ubiquitin-like protein (Ulp) domain. SENP family consists of 6 isoforms (SENP1-3, SENP5-7 in human), representing high homology with yeast Ulp1 and 2. Yeast Ulp1 deletion mutant is lethal whereas Ulp2 deletion in yeast could survive with abnormal growth and hypersensitivity to DNA damage. Each human isoform has been categorized by its enzymatic activity regarding SUMO maturation, isopeptide cleavage, and their activity towards SUMO isoforms. SENP1 and 2 predominantly revealed substrate preference to all SUMO isoforms whereas SENP3 and 5 showed a preference to only SUMO2 and 3. SENP6 and 7 have preferentially modified SUMO2/3 conjugates. Unlike other SENPs, they seemed to be involved in SUMOylated chain editing rather than deconjugation pathway. C-terminal of SENPs is relatively conserved whereas N-terminal regions are variable. Thus, a less conserved N-terminal region of SENPs plays a key role in subcellular localization and substrate specificity. SENP1 has been typically observed in the nucleus although; it has both nuclear localization signal (NLS) and nuclear export signal (NES)[15]. Along with these, NLS and NES, SENP1 have also been shown to shuttle between cytoplasm and nucleus in CV-1 cells[16]. SENP2 also contained both NLS and NES, in the nuclear envelope[17]. Later, SENP2 was discovered shuttling between cytoplasm and nucleus. Interestingly, cytoplasmic SENP2 showed polyubiquitination leading to 26S proteasome degradation[18]. Interestingly, SENP2 splicing variants showed localizations in different subcellular areas.

Axam, a SENP2 splicing variant, is found in the nucleoplasmic face of nuclear pore complex (NPC). Further, Axam2, SuPr-1, and SENP2 splicing variants are localized in the cytoplasm and promyelocytic leukemia (PML) body of the nucleus[19]. These diverse subcellular occupancies contributed towards modulation of specific substrates so as to fine-tune regulation strategy. SENP3 and SENP5 are observed to be predominantly located in nucleolus without any limitation in subcellular localization[20]. Shuttling of SENP5 between nucleus and cytoplasm, or between nucleus and mitochondria were known to be in a cell cycle-dependent manner[21]. SENP6 was primarily detected in the cytoplasm, but a recent report showed its existence in the nucleus too[22]. The complexity of subcellular localization suggested that spatial and temporal process of deSUMOylation might be critical in the regulation of SUMOylated proteins functions.

### Biologic significance of SUMO pathway

Most components of SUMOylation associated with conjugation and de-conjugation are localized in the nucleus. Therefore, their functions were relatively well understood in the cell cycle, DNA repair, transcriptional regulation, and chromatin remodeling[23]. Recent studies have proposed an additional role of SUMOylation process outside of the nucleus. For instance, GLUT1 and GLUT4 (glucose transporters) SUMOylation in plasma membrane leading to opposite outcomes regarding transporter activity[24]. A variety of targets of SUMOylation suggested that SUMO-dependent modification is tightly associated with a broad spectrum of biological events including apoptosis, genomic stability, gene expression, metabolism, signaling cascade, cell cycle, ion channel and mitochondrial dynamics[25].

### SUMO2/3

Each SUMO isoform represents substrate specificities, although there is an overlapped preference to substrates. Since identified in yeast genetics, genes associated with SUMO and its related components are critical for cellular homeostasis in mammals[26]. Haploinsufficiency of murine SUMO1 suggested its involvement in human cleft lip and palate through modification of Eya1[27]. In contrast, another study showed that genetic ablation of SUMO1 in mice did not reveal any lethality. This confirmed the redundant role of SUMO2/3 for the SUMO1 target[28]. Further, during normal conditions, SUMO2/3 is detected as unconjugated form; however, it is detected as a conjugated form during various stresses like heat shock, oxidative stress, and ethanol exposure[29]. So, it is quite obvious that SUMO2/3 might have more active response upon environmental stimulation than those of SUMO1. Moreover, the SUMO consensus motif in the N-terminal region (Lys 11) made SUMO2/3 distinct from SUMO1[30]. PolySUMOylation by SUMO2/3 has fa-

cilitated ubiquitin-mediated degradation including PML by activation of RNF4, ubiquitin E3 ligase[31] and BMAL[32]. These observations indicated SUMO2/3-dependent conjugation mediates distinctive biological functions. Further, a recent study put light on SUMO2/3 along with chromatin remodeling[33]. SUMOylation of CoREST1, a transcriptional cofactor by SUMO2/3 suggested that SUMO2/3 might participate in gene regulation[33].

### SENP5

SENP5 has both C-terminal hydrolase activities for maturation of SUMO isoforms and isopeptidase activity for SUMO deconjugation. In spite of its dual enzymatic activity, it has a preference of SUMO3 hydration and SUMO2/3 deconjugation *in vitro*[34]. Strong endopeptidase activity of SENP5 indicated that SENP5 might play a role in SUMOylation repression[35]. Although SENP5 has dual enzymatic activities, it showed fewer isopeptidase activities in comparison to SENP2. Further, Drp-1, a mitochondrial fission protein was observed to be modified by SUMO1, but got de-conjugated by SENP5[36]. Most components of SUMO pathway are noticed in the nucleus and are best characterized for nuclear proteins[37]. SENP5 is often detected in the cytoplasm of tumor cells, but normal epithelial cells also showed its equal distribution in both nucleus and cytoplasm[38]. Moreover, mitochondrial fractions from COS-7 cells have reasonable levels of SENP5. So, SENP5 shuttle between nucleus and mitochondria are involved in subcellular-specific modulation of targets.

### Congestive cardiomyopathy

The heart is a muscle organ to pump blood via the circulatory system for oxygen and nutrients delivery. Cardiomyocytes generate most of the contractile powers in hearts by well-organized contractile units called sarcomeres. Further, the heart loses cell proliferation ability soon after birth, although a recent report has discovered a very low level of cardiomyocyte turnover throughout life[39-40]. Thus, it could respond only by increasing cardiomyocyte size to improve workloads. Initially, cardiac hypertrophy is beneficial because it is an adaptive response to reduce overloaded stress, but sustained hypertrophic growth finally caused heart failure and sudden death. The heart develops hypertrophic growth in response to pathologic stimuli, leading to fibrosis, dilation, ventricular remodeling and impaired cardiac output[41]. In contrast, there is a physiologic hypertrophy that occurs as an adaptive response to continuous physiologic stresses like exercise and pregnancy.

Physiologic hypertrophy is fundamentally different from pathologic hypertrophy and has beneficial response to maintain stable cardiac function through specific molecular pathways. Pathologic hypertrophy is characterized by several alterations. Firstly, sarcomeric proteins including α-actinin are rearranged to strengthen contractile power, of which failing hearts have a reduced level. Secondly, the expression of fetal cardiac genes is reactivated. Fetal gene reprogramming includes atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and skeletal α-actin (Sk-actin)[42]. Thus, fetal cardiac gene expression is used as an indicator of pathologic hypertrophy in spite of altered expression levels of genes encoding calcium handling proteins and collagen I. Thirdly, the energy source is shifted from fatty acid to glucose. Cardiomyocytes use glycolysis to obtain main energy for proliferation during embryogenesis but after birth, fatty acid oxidation becomes metabolic pathway due to high mitochondrial capacity. This switch is often broken in pathologic cardiac hypertrophy[43]. Protein synthesis rate is also elevated to satisfy the need for hypertrophied cardiomyocytes. All alterations are adopted to elevate workloads as compensatory mechanisms. However, prolonged adaptive responses lead to de-compensatory dilated cardiomyopathy, and heart failure, which is associated with thin ventricular walls through massive cardiac muscle cell loss. The above view is controversial, but cardiomyocyte loss is sufficient to induce heart failure as observed in a recent study[44]. Further, it is noticed that human heart failure led to the elevation of apoptotic cardiomyocyte death[44]. Moreover, genetically enhanced caspase-8 in mouse heart also showed dilated cardiomyopathy with exaggerated apoptosis[45]. However, mitochondrial independent cell death pathway is another cause for the heart failure that utilizes Calpain-Calpastatin system.

Calpains are Ca2+-activated cysteine proteases[46], leading to protein degradation. Calpains affect many biologic processes including cell cycle, migration, differentiation and apoptosis[47]. The stress-induced Ca2+ influx in cardiomyocytes activated Calpains result in cleavage of pro-caspase12 leading to the activation of caspase cascade[48]. Caspase-independent cell death pathway is also emerging and apoptosis-inducing factor (AIF) is

one of the key proteins in this pathway. Originally, it was thought that AIF in the mitochondrial inner membrane is critical for mitochondrial respiratory complex I integrity, representing NADH oxidoreductase and peroxide scavenging activities[49]. N-terminal domain of AIF contained NADH and FAD, critical for mitochondrial respiratory function. Interestingly, AIF is also associated with caspase-independent cell death. AIF-mediated death function depends on elevated intracellular Ca2+ concentration that triggers mitochondrial membrane depolarization. This leads to Calpain1 activation, resulting in proteolytic cleavage. C-terminal domain of AIF is important for integrity and nuclear translocation[50]. Nuclear AIF is not so well studied but its involvement in chromosome breakage and chromatin condensation is confirmed[51].

## Conclusions

We can conclude that SUMOylation is evolving in the area of cardiology and further studies in the near future would result in better therapeutics.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

## References


1) Chen HY, Chen RH. Cullin 3 ubiquitin ligases in cancer biology: functions and therapeutic implications. Front Oncol 2016; 6: 113.
2) Mahajan R, Delphin C, Guan T, Gerace L, Melchior F. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell 1997; 88: 97-107.
3) Matunis MJ, Coutavas E, Blobel G. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. J Cell Biol 1996; 135: 1457-1470.
4) Bedford L, Lowe J, Dick LR, Mayer RJ, Brownell JE. Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. Nat Rev Drug Discov 2001; 10: 29-46.
5) Johnson ES. Protein modification by SUMO. Annu Rev Biochem 2004; 73: 355-382.
6) Wilkinson KA, Henley JM. Mechanisms, regulation and consequences of protein SUMOylation. Biochem J 2010; 428: 133-145.
7) Meulmeester E, Melchior F. Cell biology: SUMO. Nature 2008; 452: 709-711.
8) Johnson ES, Blobel G. Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J Biol Chem 1997; 272: 26799-26802.
9) Rytinki MM, Kaikkonen S, Pehkonen P, Jaaskelainen T, Palvimo JJ. PIAS proteins: pleiotropic interactors associated with SUMO. Cell Mol Life Sci 2009; 66: 3029-3041.
10) Cohen P, Tcherpakov M. Will the ubiquitin system furnish as many drug targets as protein kinases? Cell 2010; 143: 686-693.
11) Kerscher O. SUMO junction-what's your function? New insights through SUMO-interacting motifs. EMBO Rep 2007; 8: 550-555.
12) Ma G, Li S, Han Y, Li S, Yue T, Wang B, Jiang J. Regulation of smoothened trafficking and hedgehog signaling by the SUMO pathway. Dev Cell 2016; 39: 438-451.
13) Song J, Durrin LK, Wilkinson TA, Krontiris TG, Chen Y. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. Proc Natl Acad Sci U S A 2004; 101: 14373-14378.
14) Hecker CM, Rabiller M, Haglund K, Bayer P, Dikic I. Specification of SUMO1- and SUMO2-interacting motifs. J Biol Chem 2006; 281: 16117-16127.
15) Gong L, Millas S, Maul GG, Yeh ET. Differential regulation of sentrinized proteins by a novel sentrin-specific protease. J Biol Chem 2000; 275: 3355-3359.
16) Kim YH, Sung KS, Lee SJ, Kim YO, Choi CY, Kim Y. Desumoylation of homeodomain-interacting protein kinase 2 (HIPK2) through the cytoplasmic-nuclear shuttling of the SUMO-specific protease SENP1. FEBS Lett 2005; 579: 6272-6278.
17) Hang J, Dasso M. Association of the human SUMO-1 protease SENP2 with the nuclear pore. J Biol Chem 2002; 277: 19961-19966.
18) Itahana Y, Yeh ET, Zhang Y. Nucleocytoplasmic shuttling modulates activity and ubiquitination-dependent turnover of SUMO-specific protease 2. Mol Cell Biol 2006; 26: 4675-4689.
19) Best JL, Ganiatsas S, Agarwal S, Ch angou A, Salomoni P, Shirihai O, Meluh PB, Pandolfi PP, Zon LI. SUMO-1 protease-1 regulates gene transcription through PML. Mol Cell 2002; 10: 843-855.
20) Gong L, Yeh EL. Characterization of a family of nucleolar SUMO-specific proteases with preference for SUMO-2 or SUMO-3. J Biol Chem 2006; 281: 15869-15877.
21) Zunino R, Schauss A, Rippstein P, Andrade-Navarro M, McBride HM. The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. J Cell Sci 2007; 120: 1178-1188.
22) Mukhopadhyay D, Ayaydin F, Kolli N, Tan SH, Anan T, Kametaka A, Azuma Y, Wilkinson KD, Dasso M. SUSP1 antagonizes formation of highly SUMO2/3-conjugated species. J Cell Biol 2006; 174: 939-949.
23) Wang J, Schwartz RJ. Sumoylation and regulation of cardiac gene expression. Circ Res 2010; 107: 19-29.
24) Giorgino F, de Robertis O, Laviola L, Montrone C, Perrini S, McCowen KC, Smith RJ. The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. Proc Natl Acad Sci U S A 2000; 97: 1125-1130.

25) Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol 2007; 8: 947-956.
26) Jin ZL, Pei H, Xu YH, Yu J, Deng T. The SUMO-specific protease SENP5 controls DNA damage response and promotes tumorigenesis in hepatocellular carcinoma. Eur Rev Med Pharmacol Sci 2016; 20: 3566-3573.
27) Alkuraya FS, Saadi I, Lund JJ, Turbe-Doan A, Morton CC, Maas RL. SUMO1 haploinsufficiency leads to cleft lip and palate. Science 2006; 313: 1751.
28) Evdokimov E, Sharma P, Lockett SJ, Lualdi M, Kuehn MR. Loss of SUMO1 in mice affects RanGAP1 localization and formation of PML nuclear bodies, but is not lethal as it can be compensated by SUMO2 or SUMO3. J Cell Sci 2008; 121: 4106-4113.
29) Saitoh H, Hinchey J. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem 2000; 275: 6252-6258.
30) Tatham M, Jaffray HE, Vaughan OA, Desterro JM, Botting CH, Hay RT. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. J Biol Chem 2001; 276: 35368-35374.
31) Weisshaar SR, Keusekotten K, Krause A, Horst C, Springer HM, Gottsche K, Dohmen RJ, Praefcke GJ. Arsenic trioxide stimulates SUMO-2/3 modification leading to RNF4-dependent proteolytic targeting of PML. FEBS Lett 2008; 582: 3174-3178.
32) Lee J, Lee Y, Lee MJ, Park E, Kang SH, Chung CH, Lee KH, Kim K. Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. Mol Cell Biol 2008; 28: 6056-6065.
33) Ouyang J, Shi Y, Valin A, Xuan Y, Gill G. Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex. Mol Cell 2009; 34: 145-154.
34) Di Bacco A, Ouyang J, Lee HY, Catic A, Ploegh H, Gill G. The SUMO-specific protease SENP5 is required for cell division. Mol Cell Biol 2006; 26: 4489-4498.
35) Mikolajczyk J, Drag M, Bekes M, Cao JT, Ronai Z, Salvesen GS. Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. J Biol Chem 2007; 282: 26217-26224.
36) Harder Z, Zunino R, McBride H. Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol 2004; 14: 340-345.
37) Choi SJ, Chung SS, Rho EJ, Lee HW, Lee MH, Choi HS, Seol JH, Baek SH, Bang OS, Chung CH. Negative modulation of RXRalpha transcriptional activity by small ubiquitin-related modifier (SUMO) modification and its reversal by SUMO-specific protease SUSP1. J Biol Chem 2006; 281: 30669-30677.
38) Ding X, Sun J, Wang L, Li G, Shen Y, Zhou X, Chen W. Overexpression of SENP5 in oral squamous cell carcinoma and its association with differentiation. Oncol Rep 2008; 20: 1041-1045.
39) Kajstura J, Rota M, Cappetta D, Ogorek B, Arranto C, Bai, Y. Cardiomyogenesis in the aging and failing human heart. Circulation 2012; 126: 1869-1881.
40) Kose M, Akpinar TS, Bakkaloglu OK, Tufan A, Sumnu A, Emet S, Kocaaga M, Erk O, Kayacan MS, Güler K, Demirel AS. Association of genetic polymorphisms with endothelial dysfunction in chronic heart failure. Eur Rev Med Pharmacol Sci 2014; 18: 1755-1761.
41) Van Berlo, JH, Maillet M, Molkentin JD. Signaling effectors underlying pathologic growth and remodeling of the heart. J Clin Invest 2013; 123: 37-45.
42) Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. N Engl J Med 1999; 341: 1276-1283.
43) Lopaschuk GD, Jaswal JS. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J Cardiovasc Pharmacol 2010; 56: 130-140.
44) Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA. Apoptosis in the failing human heart. N Engl J Med 1997; 336: 1131-1141.
45) Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, Factor SM, Shirani J, Armstrong RC, Kitsis RN. A mechanistic role for cardiac myocyte apoptosis in heart failure. J Clin Invest 2003; 111: 1497-1504.
46) Huang Y, Wang KK. The calpain family and human disease. Trends Mol Med 2001; 7: 355-362.
47) Sorimachi H, Ishiura S, Suzuki K. Structure and physiological function of calpains. Biochem J 1997; 328: 721-732.
48) Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 2004; 4: 966- 977.
49) Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N. Kroemer G. Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett 2000; 476: 118-123.
50) Boujrad H, Gubkina O, Robert N, Krantic S, Susin SA. AIF- mediated programmed necrosis: a highly regulated way to die. Cell Cycle 2007; 6: 2612-2619.
51) Susin SA, Daugas E, Ravagnan L, Samejima k, Zamzami, M, Loeffler P. Two distinct pathways leading to nuclear apoptosis. J Exp Med 2000; 192: 571-580.