Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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Chapter 3

Anti-Carbamylated protein antibodies precede disease onset in monkeys with collagen-induced arthritis

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As published in Arthritis Res Ther. 2017 Nov 2;19(1):246.

Rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies are rheumatoid arthritis (RA)-associated autoantibodies. Besides their presence in human sera, anti-CarP antibodies have also been described in rodent models of arthritis, while ACPA are not consistently detectable. Data on these RA-associated autoantibodies in primates is still incomplete. Therefore, we investigated the presence of RF, anti-CarP antibodies and ACPA in rhesus monkeys before and after collagen-induced arthritis immunizations.

Arthritis was induced in groups of Rhesus monkeys by immunisation with collagen following pre-treatment with either placebo, abatacept or roactemra. Autoantibodies were measured by ELISA, detecting anti-CarP antibodies, RF-IgM and antibodies against CCP2, citrullinated myelin basic protein and citrullinated fibrinogen.

Out of the three autoantibodies, only anti-CarP antibodies were detectable in resus monkeys with arthritis. RF-IgM and ACPA were undetectable and below the detection limit of the ELISAs. The level of anti-CarP antibodies increases over time and, similar to human and mice, these autoantibodies were detectable already before clinical disease onset. Furthermore, preventive treatment with abatacept (CTLA4/IgG1-Fc fusion protein) inhibited the development of anti-CarP antibodies after immunization, while this was less evident for preventive roactemra (anti-IL6-receptor) treatment. Moreover, disease progression was only reduced following abatacept treatment.

In conclusion, rhesus monkeys develop anti-CarP antibodies upon induction of collagen-induced arthritis, while we were unable to detect RF of ACPA. Also, the development of anti-CarP antibodies could be inhibited by preventive abatacept treatment.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized, among others, by the presence of autoantibodies. Two such autoantibodies, rheumatoid factor (RF) and anti-citrullinated proteins antibodies (ACPA) have been incorporated into the classification criteria for RA[1]. Other autoantibodies have been identified more recently[2], including anti-carbamylated protein antibodies (anti-CarP)[3]. These antibodies target carbamylated proteins in which a lysine has been converted into a homocitrulline under the influence of cyanate. ACPA on the other hand target citrulline, which arise after enzymatic conversion of arginine[4].

Anti-CarP antibodies have been detected in sera of RA patients in several cohorts around the world and were shown to associate with the development of joint damage[3,5-8]. These antibodies could be detected several years before disease onset and associate with development of RA in arthralgia patients[5,9,10]. Furthermore, anti-

CarP antibodies can recognize multiple carbamylated antigens, such as fibrinogen and alpha-1 antitrypsin[3,11]. Anti-CarP antibodies were also detected in different rodent models for arthritis[12,13]. RF(-like) antibodies have also been described in both rodents and human[14]. While the presence of ACPA is evident in RA, they are largely undetectable in rodents[1,3,12,15].

The combination of these autoantibodies has not been measured in primates yet. We now used the collagen-induced arthritis (CIA) model in rhesus monkeys to establish the occurrence of these autoantibodies in arthritis This model was previously established for the investigation of RA and a large amount of clinical parameters has already been established[16]. To investigate RA-associated autoantibodies in the primate CIA model, we first determined the presence of ACPA, RF and anti-CarP antibodies. Second, we investigated the effect of two different treatments, roactemra (anti-IL6-receptor) and abatacept (CTLA4-IgG1 Fc fusion protein) on the clinical score and autoantibody development after immunization.

Materials and Methods

Animals

This study was conducted at the Biomedical Primate Research Centre (BPRC; Rijswijk, the Netherlands) in accordance with the Dutch law on animal experimentation. The study protocol and experimental procedures were approved by the Experimental Animal Care and Use Committee of the BPRC. CIA-susceptible adult, healthy rhesus monkeys (Macaca mulatta; BPRC) were selected based on the absence of the dominant major histocompatibility complex class I resistance marker Mamu-B26[17]. Individual data are shown in table 1.

Treatment conditions

The data was obtained from 2 studies reported elsewhere[18,19]. The first study[18] contained a group that was placebo-treated (N = 5) and a roactemra-treated (10 mg/kg; N = 7) group. The second study[19] contained a group that was placebo-treated (N = 5) and an abatacept-treated (10 mg/kg; N = 7) group. In both studies, CIA was evoked by immunization with 5mg of chicken type II collagen (MD biosciences, St Paul, MN) as before. The test substances were administered as described[18,19] (The ethical permits for these studies were obtained under number #633 and #695).

Clinical evaluation

For the clinical and ethical management signs of clinical arthritis, soft tissue swelling and redness of affected joints, were scored twice weekly using a previously published semi-quantitative clinical score[20]. To ensure objective clinical scoring the investigators performing the physical examination and rating clinical scores were blinded to the different treatments during the *in vivo* part of the study.

Study	Treatment	Animal ID	Gender	Age	Starting weight
1	Group I (Placebo)	95020	M	14.8	10.2
		R05029	M	4.9	6.1
		R05053	M	4.8	7.8
		R05058	M	4.8	7.3
		R05073	M	4.8	6.6
	Group II (TCZ)	95031	M	14.8	12.7
		BB226	M	6.8	9.9
		R04042	M	5.9	7.5
		R05059	M	4.8	6.9
		R05061	M	4.8	7.2
		R05089	F	4.8	5.1
		R05090	M	4.7	8.0
2	Group III (Placebo)	R00062	F	12.2	5.2
		R02049	F	10.3	7.7
		R05068	F	7.3	5.9
		R06045	M	6.3	7.2
		R07111	M	5.2	5.6
	Group IV (Abatacept)	96089	F	16.3	8.5
		R01091	F	11.1	5.0
		R05084	F	7.3	6.9
		R07003	M	5.5	6.5
		R07031	F	5.4	4.6
		R07068	M	5.3	7.6
		R07075	F	5.3	5.2

Table 1 - Animal identification, gender, age and starting weight at day of stratification. TCZ: tocilizumab

Measurement of anti-CarP antibodies

Anti-CarP-IgG antibodies were measured as described previously, using carbamylated fetal calf serum as antigenic target[3]. ACPA were measured using an in-house assay with CCP2 peptide, citrullinated fibrinogen(cit-fib, Sigma) or myelin basic protein(cit-MBP, Sigma) as antigen. For each antigen, both the modified and non-modified version were taken along.

For CCP2, streptavidin (Invitrogen) was coated on a plate at 5µg/ml in a carbonate buffer with a pH of 9.6 and incubated at 4 ˚C overnight. Peptides were coated at 1µg/ml in PBS with 1% BSA (Sigma) and allowed to bind for 1 hour at room temperature. Serum samples were diluted 50x in PBS with 1%BSA and 0.05% Tween (Sigma) and incubated for 1 hour at 37˚C.

For Cit-Fib and Cit-MBP, the proteins were coated at 10µg/ml in a 9.6 pH carbonate buffer and incubated overnight at 4˚C. Plates were blocked for 1 hour at 37 ˚C with PBS 2% BSA, pH 9.0. Samples were diluted 50x in RIA buffer and incubated for 1 hour at 37˚C.

For the anti-CarP and anti-citrulline ELISAs, antibody binding to the target was detected using rabbit anti-human IgG-HRP(DAKO, P0214), which is also cross-reactive for the IgG antibodies from rhesus monkeys.

RF-IgM antibodies were also measured with an in-house ELISA, using rabbit IgG (Sigma) as antigen which was diluted to 10µg/ml in carbonate buffer, pH9.6 and incubated overnight at room temperature. Plates were blocked for 1 hour at 37˚C with PBS, 1% BSA. Serum samples were diluted 100x in PBS, 1% BSA and 0.05% Tween and incubated for 1 hour at 37˚C. Antibody binding was detected with goat-anti-human-IgM-HRP(Life technologies, 627520). Cross-reactivity of this antibody was confirmed by measuring anti-collagen IgM antibodies in a selection of the monkey serum samples.

All washing steps were carried out with PBS, 0.05% Tween. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was used for final detection.

Statistics

Statistical analyses were carried out in SPSS statistics version 23 (IBM) or with Graphpad Prism version 7. To compare antibody levels over time within the same animals, a Wilcoxon rank test was carried out. To compare antibody levels between two groups, a Mann-Whitney U test was carried out. A spearman correlation was calculated to investigate a possible association between autoantibodies and clinical parameters.

Results

The presence of RA-associated autoantibodies was measured in 10 rhesus monkeys at the last two available time points after CIA-induction. Anti-CarP antibodies were readily detectable (figure 1A), while rheumatoid factor was not observed (figure 1B). ACPA were measured using 3 different antigens; CCP2 peptide, Cit-fib and cit-MBP, but no specific citrulline-directed signal could be detected (figure 1C). In summary, of the three investigated RA-associated autoantibodies, only anti-CarP antibodies but not ACPA or RF could be detected in CIA monkeys. Therefore, we further investigated the development of anti-CarP antibodies. A comparison of the anti-CarP antibodies between the time of immunization and the last available timepoint shows that the levels of anti-CarP antibodies increase over time (figure 1D). Furthermore, we observed that the anti-CarP antibodies are present before disease onset (Figure 1E). A weak correlation was observed between the presence of anti-CarP antibodies and the clinical score. A similar weak correlation could be identified between anti-CarP IgG antibodies and anti-Collagen IgG antibodies (figure F).

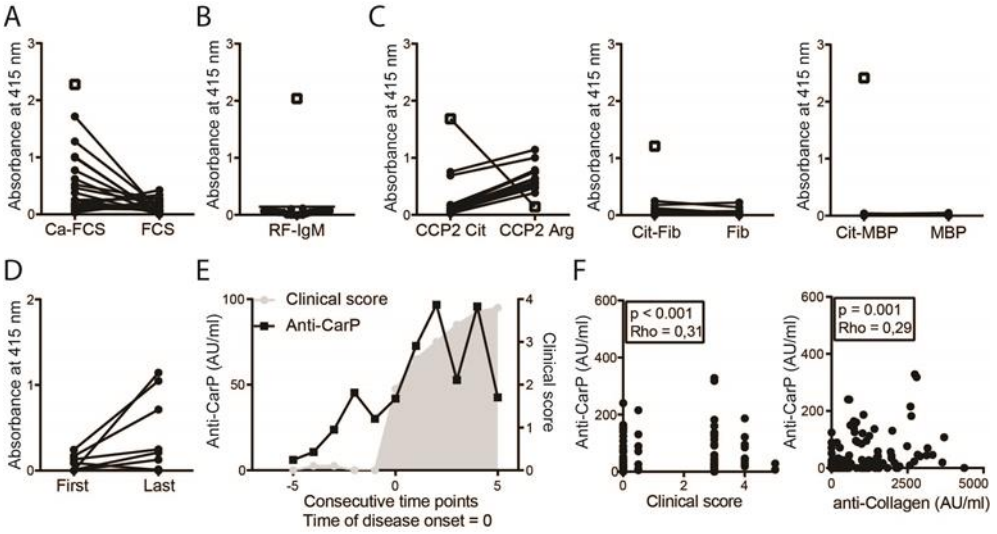

Figure 1 – Anti-CarP antibodies can be detected in rhesus monkeys, while ACPA and RF cannot.
(A) Anti-CarP IgG antibodies were measured by ELISA. Ca-FCS and the non-modified FCS served as the (control) antigen. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (B) RF-IgM antibodies were measured by ELISA, using rabbit IgG as binding antigen. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (C) ACPA IgG antibodies were measured by ELISA, using several citrullinated and control peptides or proteins as antigens. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (D) Anti-CarP IgG antibodies are shown for both the first available sample, taken at the timepoint of immunization and the last available sample are compared to each other. Antibodies were measured as in A. The absorbance values for the FCS were subtracted from the Ca-FCS absorbance values. All samples were measured on one plate. ELISAs were carried out using the 10 control monkeys who did not receive any (preventive) treatment) (E) Anti-CarP IgG antibodies and clinical score are compared over time. The point of disease onset was set at 0. Disease onset was defined as the point at which the clinical score was higher than 0.5 and increased at the next time point.
Therefore, 3 monkeys had to be excluded from this analysis. The grey area indicates the average clinical score, while the black circles show the average levels of anti-CarP antibodies at that particular time point. (F) The presence of a correlation between anti-CarP antibodies and clinical score or anti-Collagen IgG antibodies was investigated. All time points of all 10 monkeys were included for this analysis. The presence of possible associations was tested with the spearman correlation.
Anti-CarP = anti-carbamylated protein antibodies, Ca-FCS = carbamylated fetal calf serum, RF = rheumatoid factor, CCP2 = cyclic citrullinated peptide 2, cit = citrullinated, arg = arginine control, Fib = fibrinogen, MBP = myelin basic protein, AU/ml = arbitrary units per millilitre, ELISA = enzyme-linked immunosorbent assay.

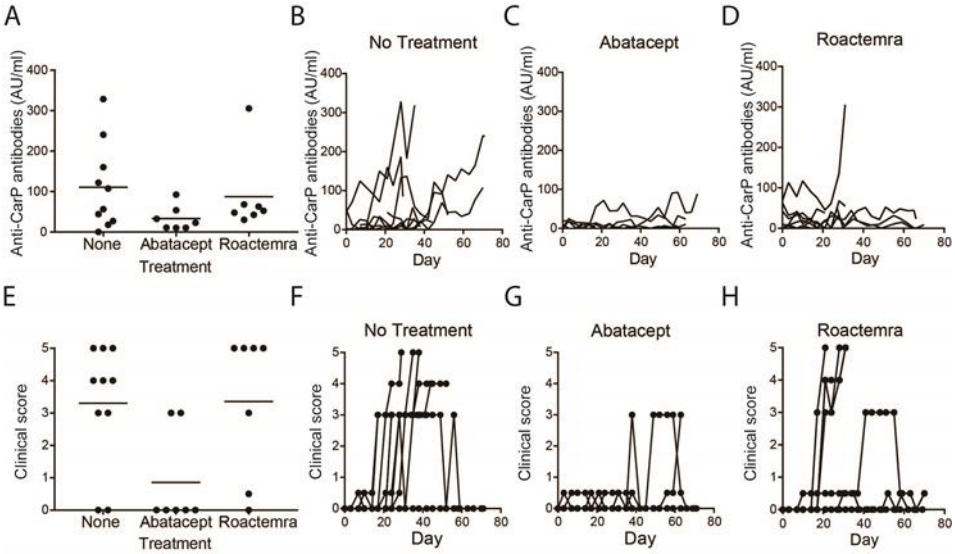

Figure 2 – Abatacept treatment reduces the development of anti-CarP antibodies and affects the severity of collagen-induced arthritis in rhesus monkeys.
(A) Anti-CarP IgG antibodies were measured by ELISA in rhesus monkeys treated with no medication (n=10), abatacept or (n=7) roactemra (n=7). The highest anti-CarP antibody level for each of the monkeys is shown. Timelines for the anti-CarP antibodies over time are shown for no treatment (B), abatacept (C) and roactemra (D). (E) The clinical score is shown in rhesus monkeys treated with no medication (n=10), abatacept or (n=7) roactemra (n=7). The highest clinical score for each of the monkeys is shown. Timelines for the clinical score over time are shown for no treatment (F), abatacept (G) and roactemra (H). Day 0 is the time point of immunization with collagen. The data in figure E-H were presented before in two separate studes [18,19] and are shown here as comparison. Anti-CarP = anti-carbamylated protein, AU/ml = arbitrary units per millilitre, ELISA = enzyme-linked immunosorbent assay.

After the detection and characterization of anti-CarP antibodies in rhesus monkeys, the effect of several (preventive) treatments on these autoantibody levels was investigated as well, using abatacept and roactemra as model treatments. Lower levels of anti-CarP antibodies are observed in the group treated with abatacept when compared to the control group without treatment (figure 2A). Although differences between the abatacept and the no treatment-group are not significant at these small group sizes, a clear trend is visible (Mann-whitney U test, p=0,087). These differences also seem to be consistent over time, showing an increase in anti-CarP antibodies in the control group, while low levels of anti-CarP antibodies were detected in the abatacept treated group (Figure 2B-D). For Roactemra, an increase in anti-CarP antibodies is only seen for one of the animals, but no statistical differences were observed between roactemra and the control group (Mann-whitney U test, p=0,89).

Furthermore, the effect of the treatments on the clinical score was investigated. Out of the three treatment groups, abatacept-treated monkeys show a reduced clinical score (Mann-whitney U test, p=0,02) (Figure 2E). The timelines show that the abatacept-treated animals do not develop full-blown arthritis, while this is not the case for the animals without treatment or for animals treated with roactemra (Figure 2F-H). No differences in clinical score were observed between the no treatment group or treatment with roactemra (mann-whitney U test, p=0,67).

Discussion

Here we have shown that anti-CarP antibodies can be present in rhesus monkeys and show that these autoantibodies are especially increased after the induction of collagen-induced arthritis. Interestingly, ACPA, which target a very similar post-translational modification as anti-CarP antibodies could not be detected in this animal model. In RA patients, there is a large overlap in the positivity for ACPA and anti-CarP antibodies and detailed studies have been performed to confirm that ACPA and anti-CarP antibody positive sera contain both cross-reactive and non-cross-reactive antibodies[3]. Now in the context of both mice[12,15] and monkeys, only anti-CarP reactivity can be observed, indicating that in these animals, anti-CarP antibodies are not cross-reactive to citrullinated proteins. In mice, ACPA have also been difficult to detect, while they are prominently present in human RA patients. The notion that ACPA are difficult to detect in both rodents and rhesus monkeys, indicates that there is a clear difference between the two autoantibodies. Furthermore, RF could not be detected in rhesus monkeys after CIA-induction conform previous data using the same model[16].

We observed a clear effect on anti-CarP antibody levels by preventive treatment with abatacept, which is a fusion protein consisting of the CTLA4-domain that can bind to CD80 or CD86 and the IgG1 Fc region[21]. This treatment is currently used in RA patients when they have failed one or more DMARDs (disease-modifying anti-rheumatic drug). In previous CIA-experiments using abatacept treatment, a general reduction of IgM and IgG antibodies was observed[19], which is in line with the reduction in anti-CarP antibodies.

Roactemra, the other intervention used, is also known under the name tocilizumab and is a monoclonal antibody that targets the IL6-receptor[22]. This is also a receptor that is involved in plasma cell development and might therefore be important for antibody production. As in RA patients, the monkeys which developed collagen-induced arthritis also showed an increase in IL-6 levels (data not shown), indicating that this disease mechanism might be similar. It is therefore not clear, why the treatment with roactemra did not have an effect on clinical score in this model.

It should also be noted that the presence of anti-CarP antibodies and clinical score did not always correlate. Some of the monkeys, did show a large increase in the levels of anti-CarP antibodies, while no arthritic symptoms were observed. Also, some animals with a higher clinical score did not develop high levels of anti-CarP antibodies. These data indicate that anti-CarP antibody levels are not directly correlated with clinical score.

All together, we conclude that abatacept treatment had a dampening effect of the antibody response in collagen-induced arthritis while also preventing disease development. Furthermore, anti-CarP antibodies, but not ACPA or RF could be detected in this animal model. Also, as observed in both mice and human, the anti-CarP antibodies presented before the actual disease onset.

References

1 Aletaha, D. *et al.* 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis* 69, 1580-1588, doi:10.1136/ard.2010.138461 (2010).

2 Trouw, L. A., Rispens, T. & Toes, R. E. Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. *Nat Rev Rheumatol*, doi:10.1038/nrrheum.2017.15 (2017).

3 Shi, J. *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* 108, 17372-17377, doi:10.1073/pnas.1114465108 (2011).

4 Malmstrom, V., Catrina, A. I. & Klareskog, L. The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting. *Nat Rev Immunol* 17, 60-75, doi:10.1038/nri.2016.124 (2017).

5 Brink, M. *et al.* Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. *Arthritis Res Ther* 17, 25, doi:10.1186/s13075-015-0536-2 (2015).

6 Montes, A. *et al.* Anti-Carbamylated Protein Antibodies as a Reproducible Independent Type of Rheumatoid Arthritis Autoantibodies. *PLoS One* 11, e0161141, doi:10.1371/journal.pone.0161141 (2016).

7 Pecani, A. *et al.* Prevalence, sensitivity and specificity of antibodies against carbamylated proteins in a monocentric cohort of patients with rheumatoid arthritis and other autoimmune rheumatic diseases. *Arthritis Res Ther* 18, 276, doi:10.1186/s13075-016-1173-0 (2016).

8 Challener, G. J. *et al.* Anti-carbamylated Protein Antibody Levels Correlate with Anti-Sa (Citrullinated Vimentin) Antibody Levels in Rheumatoid Arthritis. *J Rheumatol* 43, 273-281, doi:10.3899/jrheum.150179 (2016).

9 Shi, J. *et al.* Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. *Arthritis Rheum* 65, 911-915, doi:10.1002/art.37830 (2013).

10 Gan, R. W. *et al.* Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. *J Rheumatol* 42, 572-579, doi:10.3899/jrheum.140767 (2015).

11 Verheul, M. K. *et al.* Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis. *J Autoimmun* 80, 77-84, doi:10.1016/j.jaut.2017.02.008 (2017).

12 Stoop, J. N. *et al.* Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. *PLoS One* 9, e102163, doi:10.1371/journal.pone.0102163 (2014).

13 Turunen, S., Koivula, M. K., Risteli, L. & Risteli, J. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 62, 3345-3352, doi:10.1002/art.27644 (2010).

14 Nemazee, D. A. & Sato, V. L. Induction of rheumatoid antibodies in the mouse. Regulated production of autoantibody in the secondary humoral response. *J Exp Med* 158, 529-545 (1983).

15 Stoop, J. N. *et al.* Anticarbamylated protein antibodies can be detected in animal models of arthritis that require active involvement of the adaptive immune system. *Ann Rheum Dis* 74, 949-950, doi:10.1136/annrheumdis-2014-206797 (2015).

16 Bakker, N. P. *et al.* Experimental immune mediated arthritis in rhesus monkeys. A model for human rheumatoid arthritis? *Rheumatol Int* 10, 21-29 (1990).

17 Bakker, N. P. *et al.* Resistance to collagen-induced arthritis in a nonhuman primate species maps to the major histocompatibility complex class I region. *J Exp Med* 175, 933-937 (1992).
18 Van Roy, M. *et al.* The preclinical pharmacology of the high affinity anti-IL-6R Nanobody(R) ALX-0061 supports its clinical development in rheumatoid arthritis. *Arthritis Res Ther* 17, 135, doi:10.1186/s13075-015-0651-0 (2015).
19 Vierboom, M. P. *et al.* Clinical efficacy of a new CD28-targeting antagonist of T cell co-stimulation in a non-human primate model of collagen-induced arthritis. *Clin Exp Immunol* 183, 405-418, doi:10.1111/cei.12739 (2016).
20 Vierboom MPM, B. E., 't Hart BA. Preclinical evaluation of anti-rheumatic drugs in a non-human primate model of arthritic disease. *Drug Discovery Today: Disease Models* 89-95 (2008).
21 Keating, G. M. Abatacept: a review of its use in the management of rheumatoid arthritis. *Drugs* 73, 1095-1119, doi:10.1007/s40265-013-0080-9 (2013).
22 Oldfield, V., Dhillon, S. & Plosker, G. L. Tocilizumab: a review of its use in the management of rheumatoid arthritis. *Drugs* 69, 609-632, doi:10.2165/00003495-200969050-00007 (2009).